Neutrophil dysfunction in alcoholic hepatitis and alcohol-related cirrhosis
A contributor to immunoparesis and disease state

Ryan, Jennifer Marie

Awarding institution:
King's College London

Neutrophil dysfunction in alcoholic hepatitis and alcohol-related cirrhosis: a contributor to immunoparesis and disease state

Jennifer Marie Ryan

Thesis submitted to the Faculty of Life Sciences and Medicine at King's College London for the degree of Doctor of Philosophy

Institute of Liver Studies
School of Immunology and Microbial Sciences
Faculty of Life Sciences and Medicine
King's College London

November 2017

Abstract

Background

There has been an exponential rise in the incidence of alcohol related liver disease (ALD), particularly in the UK. Alcoholic hepatitis (AAH) is the most florid form of ALD and has a high mortality rate, sepsis is a significant problem and a major contributor to mortality. Treatment for patients with AAH has not changed in decades, one of the reasons for this is the relative lack of understanding of the underlying pathophysiology. No study has longitudinally investigated changes in immune function in this condition or the impact of current treatments on host immunity. Neutrophil dysfunction has been described in cirrhosis, however there is a lack of understanding regarding the mechanisms involved and how this relates to alcohol-induced liver toxicity. In addition, whether other derangements in neutrophil function contribute to the state of immunoparesis in ALD is not well understood.

Aims and methods

I sought to characterise neutrophil phenotype and responses *ex vivo* to bacterial challenge in patients with AAH compared with patients with alcohol related cirrhosis (ARC) and healthy controls (HC) and prospectively examine neutrophil function and the effect of current AAH therapies on innate immune function through sequential analyses. The interplay between neutrophils, ethanol and the liver was also examined by using the HL-60 cell line and creating an *in vitro* model. Finally, I sought to identify novel targets which may propagate immunoparesis in ALD, specifically examining the relationship of neutrophils and interferon-λ (IFN-λ) in anti-bacterial immune defenses in health and ARC.

Key results

Neutrophils from patients with AAH display increased reactive oxygen species production and lactoferrin release compared to ARC and HCs. Neutrophil antibacterial activities and key detecting receptors (TLRs) are dysfunctional in AAH and in ARC. Indeed, a skewed balance between host-induced immunopathology and protective anti-pathogen immunity in AAH has been confirmed. Further to this, the studies have shown that the immune impairment is reversible and that the antibacterial immune responses can be restored.

Antibodies against immunohibitory signatures, PD1 and TIM3, restored T-cell production of interferon gamma, reduced the numbers of interleukin 10-producing T cells, and increased neutrophil antimicrobial activities without exacerbating neutrophil oxidative burst.

The most notable finding in the longitudinal study was the impact of steroids on phagocytic capacity, this was significantly reduced at day 7 in the prednisolone-exposed AAH patients compared to those who did not receive prednisolone, reinforcing the concept that improved stratification for steroid-prescribing is required.

The differentiated HL-60 cells express many of the key receptors examined in the human study and could form the basis of future *in vitro* studies in this area of research. The exploration of these cells led to further questions regarding neutrophil biology and the work on the neutrophil-interferon-λ relationship both in health and ALD. The role of IFN-λ in bacterial infection was not known and the question as to whether neutrophils produce IFN-λ unanswered. Neutrophil specific IFN-λ production in response to *E. coli* challenge was found to be compromised in patients with ARC and correlated with severity of liver disease. These findings reveal a previously unknown function of neutrophils in the context of bacterial infection and identify a novel impairment in host immunity in patients with ARC.

Conclusions

Elucidation of the above, specifically the potential for immune dysfunction reversibility and the novel finding of the deficiency in neutrophil IFN-λ function, may have important implications for therapeutic developments in an era of multi-drug resistance and within the spectrum of ALD.

Table of Contents

Table of Figures

List of Tables

Abbreviations

AAH	Acute alcoholic hepatitis
ALF	Acute liver failure
ARC	Alcohol-related cirrhosis
ALD	Alcohol-related liver disease
ATRA	All-trans-retinoic acid
CARS	Compensatory anti-inflammatory response syndrome
CD	Cluster of differentiation
C.I.	Confidence intervals
CS&T	Cytometer setting and tracking
CBA	Cytometric bead array
DAMPs	Damage associated molecular patterns
DMSO	Dimethylsulphoxide
DNA	Deoxyribo nucleic acid
DNase-I	Deoxyribonuclease-I
EU/mL	Endotoxin unit / mL
FITC	Fluorescein isothiocyanate
FACS	Fluorescence activated cell sorting
fMLP	Formyl-Methionine-Leucine-Phenylalanine
FSC-A	Forward scatter channel area
G-CSF	Granulocyte colony stimulating factor
HC	Healthy controls
HE	Hepatic encephalopathy
HB	High burst
HMGB-1	High mobility group box -1
IgG	Immunoglobulin G
ICU	Intensive care unit
ICAM-1	Intercellular adhesion molecule - 1
IFNs	Interferons
IL	Interleukin
LAL	Limulus amoebic lysate
LPS	Lipopolysaccharide

LT	Liver transplantation
LB	Low burst
MFI	Mean fluorescence intensity
μM	Micromolar
mM	Millimolar
MELD	Model for end-stage liver disease
MOF	Multiple organ failure
MAL	MyD88 adaptor-like
NAC	N-Acetylcysteine
NK	Natural killer
PB	Phagoburst
NPA	Neutrophil phagocytic activity
NF-κB	Nuclear factor - κB
ODN	Oligodinucleotides
OB	Oxidative burst
PAMPs	Pathogen-associated molecular patterns
PALF	Paracetamol-induced acute liver failure
PerCP	Peridinin chlorophyll protein
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
PAG	Phosphate–activated glutaminase
PBS	Phosphate-buffered saline
PMT	Photomultiplier tubes
PE	Phycoerythrin
PMNs	Polymorphonuclear neutrophils
PI	Propidium iodide
ROS	Reactive oxygen species
T-regs	Regulatory T cells
RT	Room temperature
RPMI	Rosewell park memorial institute
SOFA	Sequential organ failure assessment
SSC-A	Side scatter channel area
SPF	Specific pathogen-free

SOB	Spontaneous oxidative burst
SIRS	Systemic inflammatory response syndrome
T_H Cells	T helper Cells
TLR	Toll-like Receptor
TIR	Toll/interleukin-1 receptor
TNF	Tumour necrosis factor
TGF	Tumorogenic growth factor
UK	United Kingdom
USA	United States of America
WBC	White blood cells

1 Introduction

1.1 Alcohol-related liver disease

1.1.1 The extent of the problem

Liver disease now constitutes the third commonest cause of premature death in the UK [1] and seventy per cent of patients, admitted with cirrhosis, have alcohol as the major aetiological factor [2]. Whilst mortality from alcohol-related liver disease (ALD) in many European nations is falling, a steady rise in alcohol related liver deaths in England and Wales is projected, estimated to reach 143,000 over the next twenty years (Figure 1.1) [3].

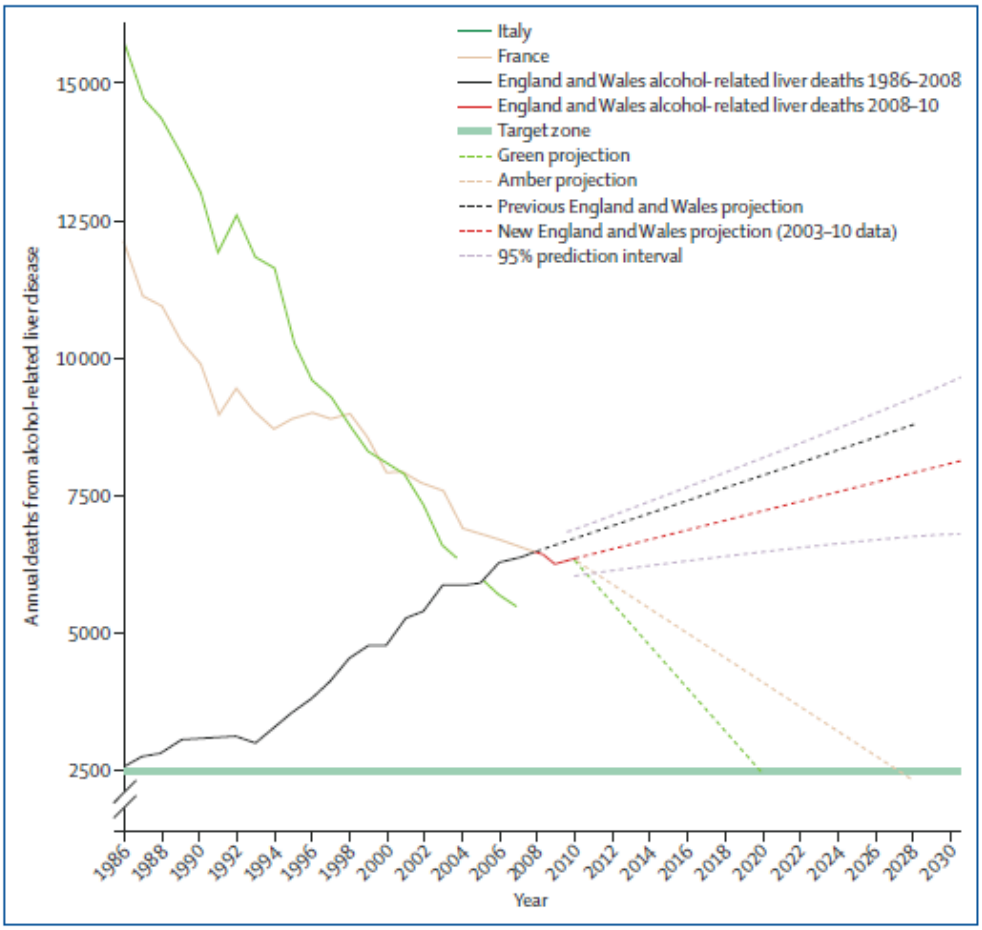


Figure 1.1 Alcohol related liver deaths by country since 1986 from Sheron *et al*, Lancet 2012 [3]

Considerable advances have been made in many aspects of healthcare over the last few decades and in some areas, particularly cardiovascular disease, the decrease in mortality has been substantial. Liver disease remains the exception.

Alcohol-related liver toxicity is dose-related and there is a significant positive correlation between overall alcohol consumption and liver related mortality in 21 out of 28 EU member states [4]. Public health measures and policy alterations have the potential to alter the predicted trend over years to come; currently we have a rising mortality from a disease where no pharmaceutical therapy has yet been shown to improve long-term survival.

In addition, the 2013 National Confidential Enquiry into Patient Outcome and Death (NCEPOD) report showed that the care of patients acutely sick with liver disease dying in hospital was judged to be good in less than half of patients. In-hospital mortality rates for cirrhosis and liver failure vary across the UK considerably (Figure 1.2) [1], with some acute trusts consistently reporting mortality rates of more than double those of the better centres. The need for improved treatments and a standardised holistic approach to care for this group of patients is clear.

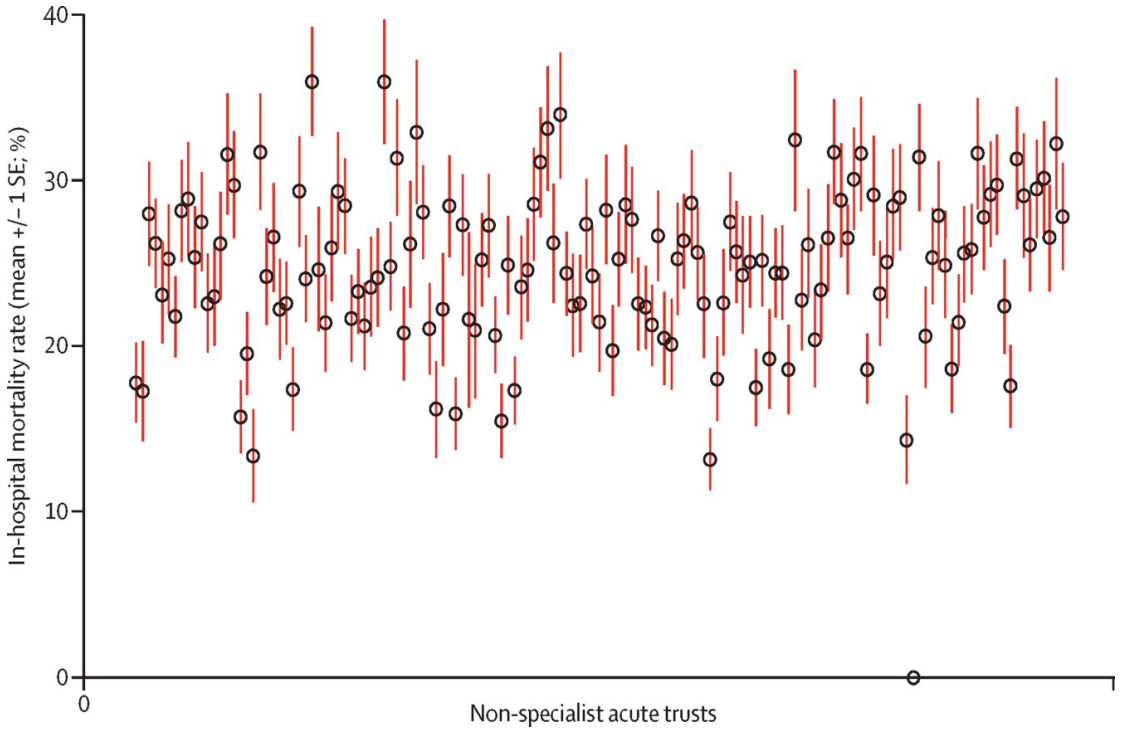


Figure 1.2 In-hospital mortality rate – patients coded for liver disease or cirrhosis in non-specialist trusts in England, 2003–13, Lancet 2014 [1]

1.1.2 Disease spectrum of alcohol-related liver disease

ALD represents a spectrum of liver injury ranging from simple steatosis, alcoholic steatohepatitis (ASH), progressive fibrosis through to cirrhosis, with the predisposition to hepatocellular carcinoma (HCC). For practical and diagnostic purposes three distinct stages are recognised:

1. Simple hepatic steatosis
2. Alcoholic steatohepatitis
3. Advanced hepatic fibrosis or cirrhosis [5].

Although many individuals drinking more than 60g of alcohol (7.5 units) per day develop steatosis, only a minority of patients with steatosis progress to ASH and 10–20% eventually develop cirrhosis [6]. Figure 1.3 illustrates the evolution of liver disease in heavy drinkers.

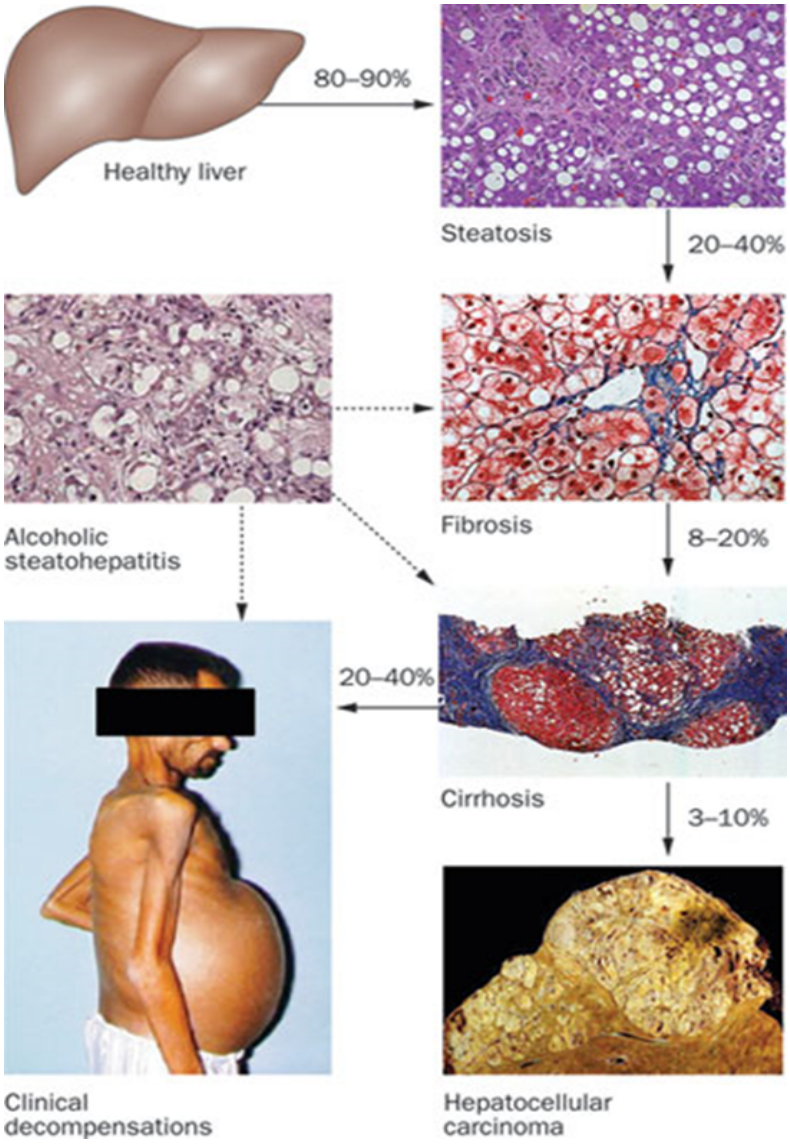


Figure 1.3 Evolution of liver disease in heavy alcohol users, adapted from Orman *et al*, Alcoholic liver disease; pathogenesis, management and novel targets for therapy [7]

Predictors for which heavy alcohol users will progress to cirrhosis are not currently well known, although it is well recognised that those with co-factors, for example, hepatitis C or obesity may run a more accelerated progressive disease course. The annual incidence of HCC in alcohol-related cirrhosis (ARC) is approximately 2.5%, even in those with compensated disease [8].

1.1.3 Alcoholic hepatitis

Alcoholic hepatitis (AAH) is a clinical syndrome defined by the recent onset of jaundice with or without other features of hepatic decompensation, including ascites, coagulopathy and encephalopathy, in a patient with recent and prolonged heavy alcohol consumption. For the diagnosis of AAH, heavy alcohol use should have occurred for >6 months, with <60 days of abstinence before the onset of jaundice [9]. ASH is a histological diagnosis and is defined by the coexistence of steatosis, hepatocyte ballooning and an inflammatory infiltrate by polymorphonuclear cells (PMN). The lesions defining ASH do not differ from those described in non-alcoholic steatohepatitis (NASH), but ASH is usually associated with more severe histological lesions and a worse clinical course. The presence of Mallory-Denk's bodies and megamitochondria are often associated with the lesions described above [10].

In most patients histological analysis demonstrates the presence of ASH and co-existent advanced fibrosis or cirrhosis.

AAH should be suspected in patients with a history of excess alcohol consumption (>60g/day male, >40g/day female) and:

- Recent onset or rapid progression of jaundice with serum bilirubin >50 μmol/L, defined as within last 8 weeks
- Leukocytosis +/- fever
- AST elevation 2-6 x ULN
- AST to ALT ratio >1.5
- Elevated GGT levels and high MCV
- Prolonged prothrombin time, decreased albumin, thrombocytopenia

Bacterial and fungal infection occurs in up to 40% [11, 12], and it is estimated that 25% of patients with AAH have active infection at the time of presentation [13]. This can be difficult to differentiate clinically from features of the systemic inflammatory response syndrome (SIRS) arising from hepatic inflammation. In addition those with underlying cirrhosis may not mount the classical immune response to sepsis. Therefore, a full septic screen (blood, urine, ascitic culture and chest X-ray) should be sent promptly on admission and at any time a new fever or clinical deterioration becomes apparent.

AAH is unlikely in the presence of very high transaminase levels (AST > 500 or ALT > 300), which is suggestive of other causes of liver disease, such as ischaemic hepatitis (e.g. due to concomitant use of cocaine), drug-induced liver injury (DILI), autoimmune hepatitis (AIH), or viral hepatitis. Similarly, high serum levels of alkaline phosphatase are unusual and suggest a possible obstructive cause of jaundice. A liver screen should be done to look for co-factors or co-existence of a second aetiology of liver disease. Imaging including

ultrasound, computed tomography or magnetic resonance imaging may allow detection of fatty infiltration and contribute to the staging of liver disease, but is particularly useful in identifying abscess, HCC or biliary obstruction which may mimic AAH [14].

The diagnosis of AAH usually can be made on clinical history, typical biochemical pattern and exclusion of other causes of acute liver injury [15]. The histological lesion (Figure 1.4) need not be accompanied by the clinical syndrome of severe AAH and ASH is often used to denote the pathological as opposed to clinical diagnosis.

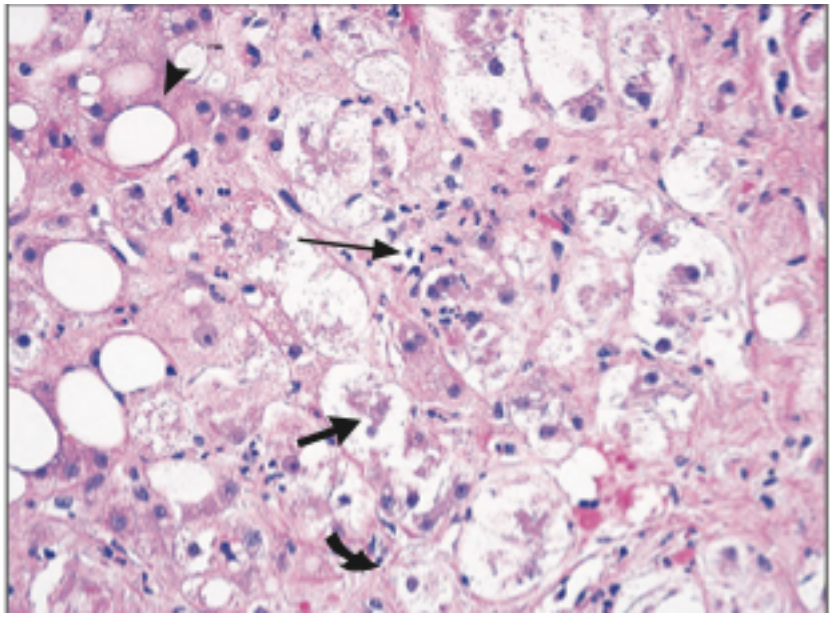


Figure 1.4 Histopathological features in a liver biopsy specimen from a patient with Alcoholic Hepatitis.

There are ballooned hepatocytes (curved arrow), some hepatocytes contain fat droplets (steatosis, arrowhead), whereas others may contain intracellular amorphous eosinophilic inclusion bodies called Mallory Denk bodies (short arrow) which are often surrounded by neutrophils (long arrow) (haematoxylin and eosin). [14]

The need for biopsy to confidently diagnose AAH remains contentious [16]. The reported inaccuracy of a clinical diagnosis of severe AAH varies widely [17, 18]. Steatohepatitis can be detected in liver tissue from approximately 80% of patients undergoing liver biopsy for presumed AAH [19, 20]. Due to the common existence of thrombocytopenia, coagulopathy and ascites in these patients, a transjugular liver biopsy (TJLB) rather than percutaneous biopsy is frequently indicated in this setting and the availability of this outside specialist centres is limited. One validated AAH-specific histologic classification has been published to date; here the degree of fibrosis, degree of neutrophil infiltration, type of bilirubinostasis, and presence of megamitochondria were independently associated with 90-day mortality [21]. The finding that neutrophil infiltration and megamitochondria identifies patients with better outcomes may relate to an earlier stage of disease in these individuals, but the protective mechanisms and potential association with regeneration requires further investigation.

Current published guidelines recommend histological confirmation of severe AAH in cases of diagnostic uncertainty or where medical therapy is contemplated and the European Association for the Study of the Liver guidelines recommend use of histologic analysis in clinical trials [5, 10].

Towards the end of my period of research the National Institute on Alcohol Abuse and Alcoholism (NIAAA) formulated definitions to specifically facilitate trial design and uniformity in clinical trials [9]. These suggest that patients for clinical trials are classified as *definite* AAH; clinically diagnosed and biopsy-proven, *probable* AAH; clinically diagnosed without any confounding factors and

possible AAH; clinically diagnosed but with confounding factors such as ischaemic hepatitis or drug-induced liver injury, uncertain alcohol use or atypical laboratory tests. It is recommended that the latter group undergo biopsy for confirmation prior to clinical trial entry.

Ultimately individual patient management with regard to biopsy is dependent on local expertise and the aim should be to assess and manage patients promptly with appropriate recognition of severity of illness.

Management currently centres around supportive care with focus on abstinence, nutrition and monitoring for sepsis. Treatment, including specific therapies, will be discussed in 1.6.

1.2 Prognostic scores in liver disease

1.2.1 Child-Pugh Score

The Child-Pugh score (CPS) was originally developed in 1973 to predict surgical outcomes in patients presenting with bleeding oesophageal varices [22]. It contains five variables including serum levels of bilirubin and albumin, prothrombin time, ascites and encephalopathy (Figure 1.5).

Many subsequent studies have shown that CPS is predictive in the assessment of prognosis in patients with liver disease. These studies demonstrated that each of the five individual clinical variables as well as the overall CPS classification had prognostic significance [23-29] (Figure 1.6).

This score has stood the test of time and continues to be used in clinical practice for patients with cirrhosis of all aetiology.

Child's grading of disease severity in chronic liver disease with Pugh's modifications			
Criteria assessed	Points scored for increasing abnormality 1	2	3
Encephalopathy (grade)	None	1–2	3–4
Ascites	Absent	Slight	Moderate
Serum bilirubin [μmol/L (mg/dL)]	<35 (<2)	35–50 (2–3)	>50 (>3)
In primary biliary cirrhosis	<70 (<4)	70–170 (4–10)	>170 (>10)
Serum albumin [g/L (g/dL)]	>35 (3.5)	35–28 (3.5–2.8)	<28 (<2.8)
Prothrombin time [prolongation (s)]	1–4	4–10	>10
Total score	5–6	7–9	10–15
Child's grade equivalent	A	B	C
Overall mortality in Pugh's series (%)	29	38	88

Figure 1.5 Child-Pugh score

Percentage survival in chronic liver disease			
Child's grade	At 1 year	At 5 years	At 10 years
A	84	44	27
B	62	20	10
C	42	21	0

Figure 4.27 Survival in chronic liver disease. The risk of death in patients with chronic liver disease correlates well with the Child's grade. (Data extracted from Christensen et al. Hepatology, 1984).

Figure 1.6 Child-Pugh score and percentage survival

1.2.2 MELD Score

The inter-observer variability for the subjective parameters in the CPS classification led to the development of the "model for end stage liver disease" (MELD) score. The MELD score was originally developed to predict survival following transjugular portosystemic shunts (TIPS) for treatment of variceal bleeding or refractory ascites [30]. It was then modified slightly to predict survival in patients with cirrhosis in general [31]. It is a continuous function of bilirubin, international normalized ration (INR) and creatinine to predict short-term (three-month) survival and has subsequently been applied to prioritise allocation of donor livers for transplantation [32].

MELD has also been used to predict mortality specifically in AAH and has been compared with Maddrey's discriminant function and CPS in this condition [33-35].

1.2.3 Maddrey's Discriminant Function

The Discriminant Function (DF), comprising bilirubin and prothrombin time (PT) was described by Maddrey in 1978 during a therapeutic trial of methylprednisolone [36] in AAH. Due to the variability in PT between laboratories the DF was modified in 1989 for a multi-centre trial to use prolongation in PT above laboratory control time [37]. A cut-off value of 32 was used to identify patients with severe AAH. By using the DF, large variations in mortality were observed between patients with DF values of 32 or more compared with patients with lower DF values [38]. Although trials pre-2000 enrolled relatively small numbers of patients, the DF remains the most widely used criteria to define severe AAH and the point at which to consider medical

therapy.

1.2.4 Glasgow Alcoholic Hepatitis Score (GAHS)

The Glasgow Alcoholic Hepatitis Score (GAHS) is based on a multivariable model that includes age, serum bilirubin, blood urea nitrogen, PT and peripheral white blood cell count [39]. An initial validation study found that a GAHS ≥9 had lower sensitivity but higher specificity for predicting 28-day mortality than a DF of ≥32 (81 versus 96 percent and 61 versus 27 percent, respectively). In a follow-up study three-month survival in cases where GAHS<9 was unchanged by corticosteroid therapy whereas those with higher scores appeared to benefit (28 day survival 78% versus 52%, 59% versus 38% at 84 days) [40].

1.2.5 The Lille score

This model specific for AAH uses five routine pre-treatment variables and change in bilirubin level at day 7 [41]. Above the cutoff of 0.45, the Lille model is able to predict 76% of the observed 6-month deaths. The absence of differences in survival among patients with a Lille score 0.45 who were treated with either corticosteroids or placebo suggests that continuing corticosteroids after 7 days may be futile.

1.2.6 ABIC score

In 2008 a further AAH-specific prognostic score was identified and this used age, bilirubin, INR and creatinine (ABIC) as independent predictors of 90 day mortality in a prospective study [42]. Using two points in the ABIC score investigators defined three groups with no (0%), moderate (32.5%) and high (72.5%) risk of death at 90 days. Admission ABIC scores remained predictive of

survival at one year and suggests that corticosteroids appear to be most efficacious in cases of intermediate disease severity.

In summary there are multiple prognostic scoring systems for use in AAH. As above, some have been developed specifically for this cohort perhaps as a consequence of the inadequacy of existing medical therapies, others, namely MELD, have been validated for prognostic use in this population. In a cross validation study of nine scoring models for mortality in AAH, MELD, DF, GAHS and the ABIC proved to be clinically useful scores, performing comparably and with an acceptable accuracy (AUROCs exceeding 0.70) for both 30- and 90-day mortality [43]. The difficulty comes in predicting longer-term outcome. In another study, MELD, DF, ABIC and GAHS performed equally in predicting short-term (30- and 90-day) survival; all models were uniformly poor in predicting longer-term (6-month and 1-year) outcome [44]. By combining MELD and Lille scores mortality may be better predicted, this applies currently to 2-month and 6-month mortality [45]. Abstinence has been found to be the only independent predictor of long-term survival [46] and a more recent study concludes that new therapeutic development for severe AAH should target liver injury in the short-term and alcohol consumption in the long term [47].

It is clear that AAH has a spectrum of severity. Consensus is needed to select the prognostic factors and the time points for measurement to make this disease area more amenable for drug development [15].

1.3 Pathogenesis of alcohol-related liver disease

The mechanisms by which long-term alcohol exposure causes liver disease are complex and not fully understood. It is likely that multiple mechanisms combine to produce the varying stages of liver injury dependent on drinking pattern, environmental and individual factors. In this section an overview of key pathways will be described.

1.3.1 Ethanol metabolism and cell injury

Ethanol is mainly metabolized by alcohol dehydrogenase (ADH) and microsomal ethanol oxidation system (MEOS) into acetaldehyde [48]. MEOS incorporates cytochromes p450 and in particular CYP2E1. Most acetaldehyde is converted into acetate by aldehyde dehydrogenase (ALDH), this reaction requires NAD+/NADH and increases the amount of NADH in the liver. ADH, CYP2E1 and ALDH are mainly expressed in hepatocytes and therefore most of the direct cellular toxicity of ethanol affects these cells. Ethanol metabolism leads to accumulation of reactive oxygen species (ROS), these radicals bind to ethanol or iron atoms to form reactive metabolites responsible for lipid peroxidation of cell membranes.

The cytotoxic effects of ethanol metabolism and ROS lead to cell death, this results in a cascade of damage-associated molecular patterns (DAMPs) release and activation of neutrophils and macrophages, fibrogenesis and hepatic regeneration [49]. Glutathione protects cells against ROS but chronic ethanol exposure leads to glutathione depletion.

There is also enhanced endoplasmic reticulum (ER) stress, and inflammation is compounded by stimulation of the interferon and NF-kB pathways [50]. Another

consequence of ER stress is the activation of steatogenic pathways in hepatocytes [51].

1.3.2 Alcohol-induced steatosis

Chronic alcohol consumption leads to steatosis via generation of acetaldehyde, ROS and ER stress. The consequences are blockade of peroxisome proliferator-activated receptor α (PPAR α) and of adenosine monophosphate-activated protein kinase (AMPK), which is responsibe for fatty acid oxidation and export via acetyl CoA carboxylase (ACC) and carnitine palmioyltransferase 1 (CPT1). Chronic alcohol consumption and increased tumour necrosis factor (TNF) also induces sterol regulatory element-binding protein 1c (SREBP1c) activation which is responsible for fatty acid synthesis [48]. These mechanisms lead to increased lipogenesis and decreased fatty acid oxidation and export.

1.3.3 Dysbiosis and endotoxin exposure

The role of bacterial translocation and endotoxin in the pathogenesis of ALD has been clearly established. Chronic alcohol exposure in humans and animal models increases circulating levels of lipopolysaccharide (LPS) and the severity of hepatic injury correlates with serum LPS levels [52-55]. LPS is a component of Gram-negative bacteria and, biochemically, consists of an O-antigen, a core polysaccharide and a lipid-A component [56]. After translocation from the gut lumen to the liver, LPS and other pathogen-associated molecular patterns (PAMPs) are recognized by receptors, including toll-like receptors (TLRs) activating Kupffer cells. Upon activation Kupffer cells release ROS, adhesion molecules (intracellular adhesion molecule 1, ICAM-1 and vascular cell adhesion

protein 1, VCAM-1), chemokines (IL-8, CCL2) and proinflammatory cytokines (TNF, IL-1 and IL-6) [57].

AAH is characterised by the presence of large amounts of cytokines highly sensitive to PAMPs (IL-8, Gro-α, CCL2), leading to neutrophil recruitment in the liver [58-61]. Kupffer cells can also ameliorate hepatocellular damage from alcohol, adopting an anti-inflammatory phenotype and releasing IL-10 [62-64].

The gut microbiota and susceptibility to ALD has become a focus of increased research in recent years. Animal models demonstrate leaky gut and patients have impairments to the intestinal barrier, this contributes to the development of ALD [65]. Probiotics and prebiotics have subsequently been investigated as potential therapies [66], with increasing evidence that it may be possible to prevent ALD by intestinal microbiota manipulation [67].

1.3.4 Innate immune response

Dysregulation of many components of the innate immunity in the liver is thought to contribute additively or synergistically to alcohol-induced liver injury, acceleration of viral infection and tumour formation (Figure 1.7).

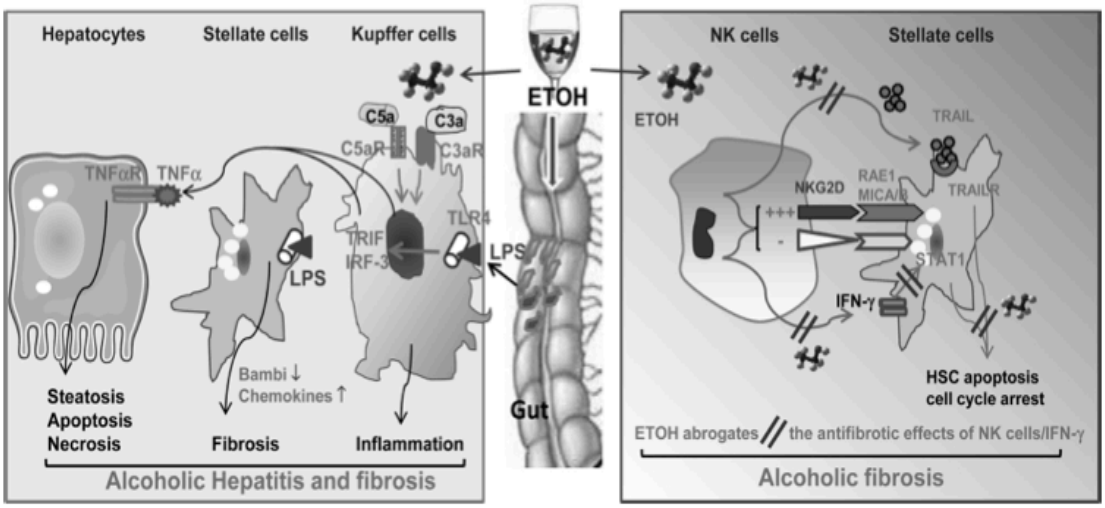


Figure 1.7 Ethanol dysregulation of innate immunity contributes to the pathogenesis of ALD.

Chronic alcohol consumption results in activation of innate immunity components such as Kupffer cells and complements or inhibition of innate immunity components such as natural killer (NK) cells. First, as previously described, alcohol consumption increases gut permeability and subsequently hepatic LPS levels via binding to TLR4; Kupffer cells are then stimulated to produce TNF-α in a TRIF/IRF-3 dependent manner. LPS can also directly target hepatic stellate cells (HSCs) and enhance TGF-β signaling and expression of chemokines, adding to liver fibrosis. Second, alcohol consumption results in activation of complement components C3a and C5a, again stimulating Kupffer cells to produce TNF-α, contributing to hepatocellular damage. Third, alcohol consumption inhibits the anti-fibrotic effects of NK cells and interferon (IFN)-γ via multiple mechanisms. NK cell functions are blocked via blocking IFN-γ and TRAIL production, IFN-γ signaling in HSCs is also inhibited and HSC are rendered resistant to NK cell killing. [68]

Kupffer cells The key role of LPS in alcohol-induced steatohepatitis is believed to be mediated via targeting TLR4 on Kupffer cells. The TLR4 adaptor molecule, MD-2, and its co-receptor, CD14 bind LPS and bring it to the receptor complex for recognition by TLR4. Hepatocytes also express TLR4 but at low levels with minimal response to LPS [69].

Two downstream signaling pathways are activated upon Kupffer cell TLR4 binding. The MyD88-dependent pathway ultimately results in activation of NF-kB and induction of NF-kB controlled genes. Second the MyD88-independent pathway results in IKK/TAK1 kinase and IRF-3 phosphorylation and late activation of NF-kB. Phosphorylated IRF-3 subsequently activates the transcription of IFN-α and β and other IFN-induced genes [56, 70].

NK cells NK cell function is thought to be suppressed in ALD, and ethanol-inhibition of these cells may play an important role in accelerating viral infection, fibrosis and HCC in patients with viral hepatitis and excess alcohol intake [68].

Neutrophils Infiltration of a large number of neutrophils is a hallmark feature of AAH however the pathogenic role of neutrophils in ALD is not fully understood. It is believed that activated Kupffer cells produce a variety of cytokines and chemokines, as previously described which subsequently attract neutrophils to the liver. Neutrophils likely contribute to hepatocellular damage by producing ROS and proteases [71]. This will be discussed in further detail in 1.5.

1.3.5 Adaptive immune response

The mechanisms by which alcohol triggers the adaptive immune system are incompletely characterised. Patients with advanced ALD have circulating IgG and T-lymphocytes which recognise epitopes derived from protein modification by

hydroxyethyl free radicals and end products of lipid-peroxidation. High titres of IgG against lipid peroxidation-derived antigens are associated with an increased hepatic production of proinflammatory cytokines/chemokines [72]. In particular, patients with AAH have increased levels of circulating antibodies against lipid peroxidation adducts and increased numbers of T cells in the liver [57].

The susceptibility to infection in this patient group has been a research area of much interest for some years. In AAH there appears to be a skewed homeostatic balance between protective anti–pathogen immunity and host-induced immunopathology. Programmed cell death 1 (PD1), the T-cell immunoglobulin and mucin domain– containing protein 3 (TIM3), and their respective ligands—CD274 (also known as PD ligand 1 [PDL1]) and galectin-9—are inhibitory receptors that regulate the balance between protective immunity and host immune-mediated damage. It has been shown that sustained hyperexpression of PD1 and its ligand PDL1 promote immune exhaustion [73, 74]. TIM3 also plays a critical role in regulating the activities of innate cells, including monocytes, dendritic cells, and NK cells to control excessive inflammatory responses that could otherwise lead to immune-mediated damage and immune suppression [75]. TIM3-blockade can reverse immune tolerance in chronic viral infections and promote the clearance of tumours in humans [76]. TIM3 and PD1 up-regulation is associated with tumour-specific T-cell dysfunction in melanoma patients, and only dual blockade of both pathways allows reversal of these defects [77]. These studies suggest that manipulation of both immune-inhibitory pathways represents a promising target for novel immunotherapeutic approaches. The role of these immune inhibitory receptors in driving immune

impairments in patients with ALD and the interplay between the innate and adaptive immune system has been investigated in this work and is discussed further in results chapter 3 [78].

1.3.6 Genetics

The heritability of alcohol dependence is well documented and although variants in ADH and ALDH have been shown to confer protection in East Asians, no strong candidate genes conferring risk have been identified. Three candidate genes with regard to alcohol-related cirrhosis, PNPLA3, TM6SF2 and MBOAT7, have been identified, however, the mechanisms by which variants in these genes confer risk and the nature of the functional interplay between them remains to be determined [79]. Further advances may be made by the GenomALC consortium which has implemented a prospective approach for an intended genome wide association studies of alcohol-related cirrhosis [80].

1.3.7 Liver regeneration

Liver regeneration is essential in restoring liver function after insults such as partial hepatectomy or toxic insult. Regeneration is of particular importance in AAH and is a key target for the development of new therapies. Models of partial hepatectomy have identified TNF, IL-6, STAT-3 and NF-kB as the key drivers of liver regeneration [48]. Data are lacking on the effect of accumulation of liver progenitor cells in alcohol-injured livers, but an incomplete differentiation process leading to repopulation by ductular cells rather than mature hepatocytes could explain why some patients with severe AAH fail to recover [81, 82]. A recent study found that the LPS-TLR4 pathway drives accumulation of KRT23-positive ductular cells [83] potentially opening up further therapeutic targets.

Mathurin and colleagues have shown that patients with severe AAH who do not respond to medical treatment have low hepatic expression of TNF and IL-6 and an aberrant regeneration process in which hepatic progenitor cells cannot differentiate into mature hepatocytes [84]. Neutrophil infiltration is a hallmark of AAH and is believed to contribute to hepatocellular damage and inflammation. However, a recent study reported that infiltration of neutrophils is associated with better prognosis in AAH, suggesting that neutrophils may also play beneficial roles in promoting liver repair [21]. The presence of neutrophils and, by inference, active inflammation may indicate an early stage of disease; and those with more advanced forms of AAH are characterised by poor regenerative response [82]. Clearly neutrophils are involved in driving the process at the outset but once AAH is established they may have additional roles in relation to regeneration which are yet to be recognised. Again, some of the difficulty here relates to when patients present and the lack of studies investigating sequential biopsies and histological evolution. Neutrophils are discussed further in 1.5.

It is likely that further information regarding histological characteristics and regenerative potential/survival will be published and further individualise patient management in the future.

1.3.8 Animal models

There is a lack of animal models that mimic the entire spectrum of ALD seen in humans. The first model of chronic alcohol exposure was developed in rats and induced isolated steatosis [85]. This was then modified so that larger quantities of ethanol could be administered for 4-12 weeks, the Lieber-De-Carli model [86]. Animal models with severe inflammatory lesions do exist (the chronic plus binge

drinking model [87] and the modified Tsukamoto-French model [88]) but these do not have underlying cirrhosis or bilirubinostasis. Extrapolation from these models is clearly useful, but data from human samples are the best way to further the understanding of the pathogenesis and treatment of ALD [48].

1.4 Sepsis in alcohol-related liver disease

1.4.1 Prevalence of infection and impact on mortality in cirrhosis

Bacterial infections are more common in patients with cirrhosis than in the general population [89] and infection is more common in decompensated cirrhosis than in compensated cirrhosis [90]. Infections are present at admission or develop during hospitalization in 25-35% of patients [89, 91]. The exact mechanisms of increased susceptibility to infections in cirrhosis are unclear. It has been suggested there is a role for deficiencies in C3 and C4, down-regulation of monocyte human leukocyte antigen–DR expression and impairment of macrophage Fc-receptor–mediated clearance of antibody-coated bacteria [92]. Work in the 1980s revealed that patients with ARC have depressed neutrophil phagocytic and intracellular killing [93]. It is now thought that multiple mechanisms are likely to synergistically contribute to the increased risk of infection in cirrhosis (Figure 1.8). The aim of the work presented in this thesis is to further explore the increased susceptibility and neutrophil impairment seen both in ARC and AAH.

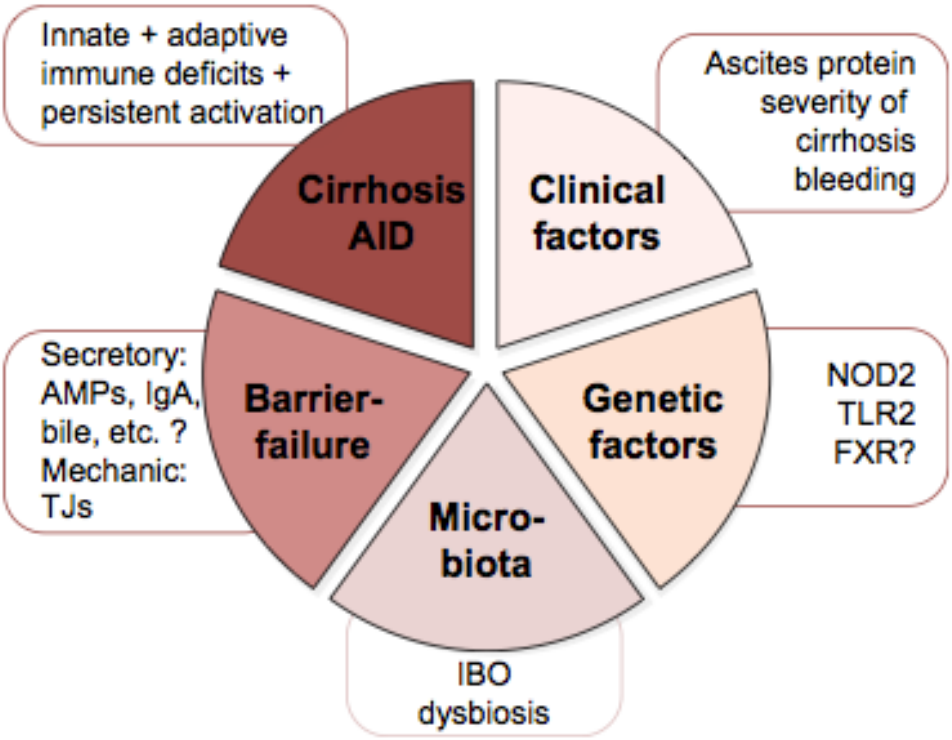


Figure 1.8 Pathogenesis of bacterial infection in cirrhosis.

The risk of infection is due to multiple factors that include liver dysfunction, porto-systemic shunting, dysbiosis and bacterial translocation, cirrhosis-associated immune dysfunction (AID) and genetic factors [94].

Bacterial infection increases the probability of death of patients with decompensated cirrhosis 3.75 fold, with mortality rates of 30% at 1 month and 63% at 1 year [95, 96]. Two important findings were highlighted by Arvaniti *et al's* review. First, for infection there was no improvement in survival over several decades; this was seen across all the subtypes of infection except spontaneous bacterial peritonitis (SBP). Second, prognosis was significantly adversely affected even after resolution of infection and several reasons were put forward to explain this, including persistent cytokine imbalance, cytokine-mediated cardiomyopathy with resultant renal dysfunction and persistently elevated hepatic venous pressure gradient following SBP [96]. Much of the literature involves study of cirrhosis of all aetiology, rather than those with ARC alone.

1.4.2 Prevalence of infection and impact on mortality in alcoholic hepatitis

Sepsis is a significant problem in AAH and impacts on mortality, much of the controversy regarding steroid use has revolved around the potential increase in susceptibility to infection following steroid treatment (discussed further in 1.7). The first study to comprehensibly address the incidence of infection in AAH found that patients with severe AAH have a higher prevalence of septic events (38% versus 25%) compared to those with advanced cirrhosis (Figure 1.9), and AAH patients with infection have significantly higher mortality compared to those without (47% versus 16%) [11].

Louvet *et al* found that nearly 25% of AAH patients are infected at admission, in addition they conclude that corticosteroid treatment is not associated with a higher risk of infection and propose that infection appears to be a consequence of absence of improvement in liver function, i.e. non-response to steroids is the

main factor contributing to the development of infection [13]. Whether steroid unresponsiveness leads to infection or whether it is the presence of infection that leads to lack of response is difficult to firmly answer. When prednisolone is not used, the effect of infection on 90-day mortality is secondary to baseline liver impairment and early improvement in liver function; patients not treated with prednisolone but who have poor liver function are more likely to develop infection and die within 90 days [97]. The persistent severe liver injury *per se* is probably the critical factor, some patients may enter a loop of persistent infection however which would clearly impact on parameters such as bilirubin, coagulopathy, encephalopathy and renal function.

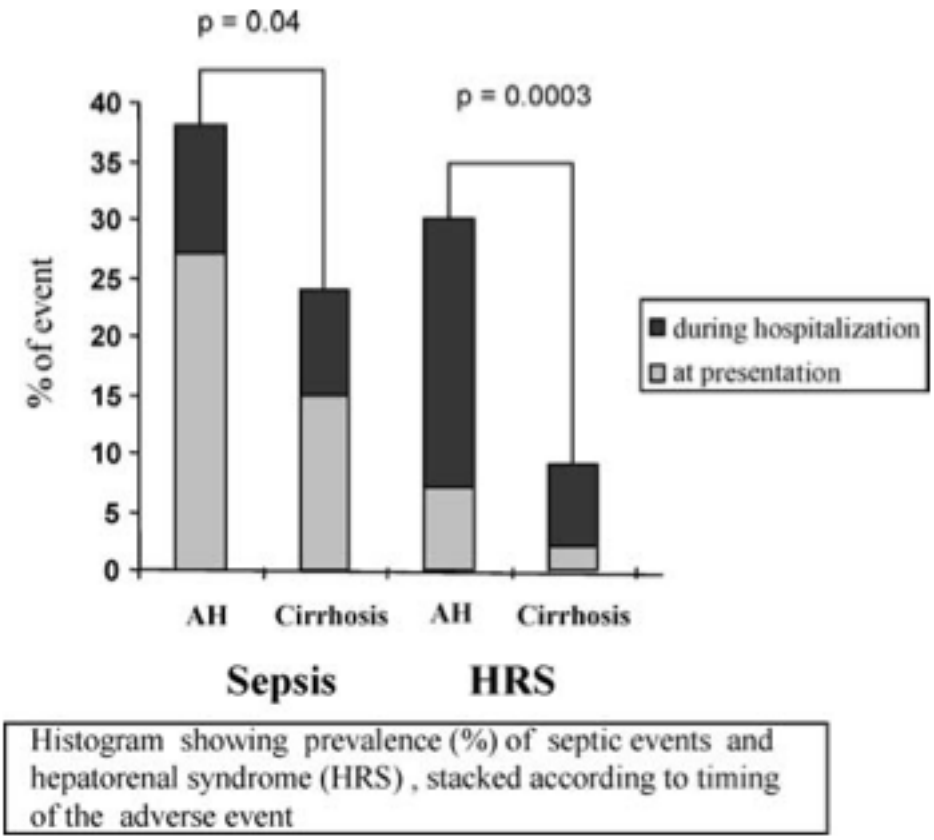


Figure 1.9 Histogram showing prevalence (%) of septic events and hepatorenal syndrome (HRS) in AH compared to cirrhosis, stacked according to timing of adverse event [11].

HRS is the development of renal failure in patients with advanced liver disease.

In addition to bacterial infection, fungal infection has also been found to be of significance in AAH. In one study invasive Aspergillosis was present in 16% of patients with severe AAH and despite antifungal treatment was a lethal complication in almost every case [12]. It often occurs at the more severe end of the spectrum and in those requiring intensive care support and has, historically, been difficult to diagnose. However there is now more awareness of the potential presence of fungal infection and new techniques in development to aid prompt diagnosis.

The Steroids or Pentoxifylline for Alcoholic Hepatitis - STOPAH study was a multicentre, double-blind, randomized trial with a 2-by-2 factorial design to evaluate the effect of treatment with prednisolone or pentoxifylline [98] (discussed further in 1.7). The primary end-point was 28 day-mortality. The incidence of infection was also studied and was an eagerly anticipated outcome given previous controversy surrounding steroid-use. Patients with a clinical diagnosis of AAH and severe disease (Maddrey's >32) were randomly assigned to one of four groups: a group that received a pentoxifylline-matched placebo and a prednisolone-matched placebo, a group that received prednisolone and a pentoxifylline-matched placebo, a group that received pentoxifylline and a prednisolone-matched placebo, or a group that received both prednisolone and pentoxifylline. Prednisolone was associated with a reduction in 28-day mortality that did not reach significance and there was no improvement in 90-day or 1-year outcome. Infection occurred in 71 of the 547 patients (13%) who received prednisolone as compared with 38 of the 545 patients (7%) who did not receive prednisolone ($p= 0.002$). Mortality attributed to infection was similar across groups but the authors do comment that infection probably played a role in

deaths attributed to other causes such as multi-organ failure. This perhaps also highlights the difficulty in diagnosing occult infection in this group.

1.4.3 Bacterial infection

SBP and urinary tract infections are the most frequent infections observed in cirrhosis, followed by pneumonia, skin infections and bacteraemia [94]. In a study of critically ill cirrhotic and non-cirrhotic patients the lungs were the most common site of infection in all patients, but abdominal infections were more common in cirrhotic than non-cirrhotic patients [99]. On review of the supplementary data from the STOPAH trial, lung infection appeared to be the most common infection [98]. Enterobacteriaceae and non-enterococcal streptococci cause the majority of spontaneous infections in patients with cirrhosis [94]. The epidemiological pattern of bacteria however differs widely among geographical areas, as do resistance patterns, and therefore local antibiotic policy derived from local patterns of infection are key in ensuring appropriate antimicrobial treatment is delivered.

1.4.4 Fungal infection

As discussed above, invasive Aspergillosis in AAH has a significant impact on mortality and is often a late diagnosis. In the study examining early liver transplantation for AAH, 26 patients underwent liver transplantation (discussed further in 1.7), five of six deaths were related to infection occurring within 2 weeks after surgery; the infection was invasive aspergillus infection in four cases [100]. There has been increasing interest in fungal infection in cirrhosis. In a study that examined a large number of cirrhotic patients admitted to the intensive care unit the incidence of invasive fungal disease was low at 1%, but

higher than in another high risk group [101]. Techniques for diagnosis are improving and fungal infection should always be considered in severe AAH, although there are no clear guidance on the role of antifungals. Neutrophils are key immune cells in combating fungal sepsis and the association between absolute or qualitative deficiency in neutrophils and the propensity for invasive fungal infection has been recognised for 50 years [102]. This, in part, explains why patients with AAH are vulnerable to fungi.

1.4.5 Multi-drug resistance

Multidrug resistant (MDR) organisms are, by definition, resistant to three or more antibiotic classes [94]. The prevalence of infections with MDR in cirrhosis has become alarmingly high and this has been seen across the globe. In series from Europe, North America and Asia, 11-45% of patients with SBP were infected with organisms resistant to first-line third-generation cephalosporins [103]. Predictors of resistance include recent and frequent antibiotic use, nosocomial acquisition of infection and recent infection with an MDR organism [89, 103, 104]. Infections due to MDR organisms are associated with an increased risk of death in both the pre-transplant and post-transplant settings [91, 103]. The impact is already substantial. Antibiotic stewardship is now adopted in UK hospitals and most liver units are guided by local epidemiology, this can, however, be difficult to track in certain centres due to transfers from widespread areas. Early de-escalation policies and short duration of antibiotic treatments in addition to restriction of antibiotic prophylaxis to only those at the highest risk of infection should be implemented to minimize the development of antibiotic resistance. Fernandez *et al* discuss the benefits and drawbacks of

prophylactic antibiotics in cirrhosis in a recent review and conclude that further development of non-antibiotic strategies based on mechanisms of infection is urgently needed [105].

1.4.6 Systemic inflammatory response syndrome and compensatory anti-inflammatory response syndrome

The concept of a systemic inflammatory response syndrome (SIRS) came from a 1991 consensus conference charged with the task of developing a set of clinical parameters to aid in the early identification of potential candidates to enter into clinical trials to evaluate new treatments for sepsis. SIRS was defined as the presence of at least 2 of the following criteria: (a) a core temperature of >38 °C or <36 °C; (b) a heart rate of ≥90 beats/minute; (c) a respiratory rate of ≥20 breaths/minute; or (d) a white blood cell (WBC) count of ≥12,000/mm^3 or ≤4000/mm^3, or a differential count showing ≥10% immature polymorphonuclear neutrophil cells (PMNC) [106].

The compensatory anti-inflammatory response syndrome (CARS) describes prolonged elevations in anti-inflammatory mediators and
immune dysregulation with defects in both the innate and adaptive
immune responses [107]. CARS has been described as a harmful sequel to severe sepsis; patients can enter into this phase of immunoparesis (Figure 1.10) and studies in patients without cirrhosis have shown that the severity of this phase determines outcome beyond the initial 'cytokine storm' associated with SIRS [108, 109].

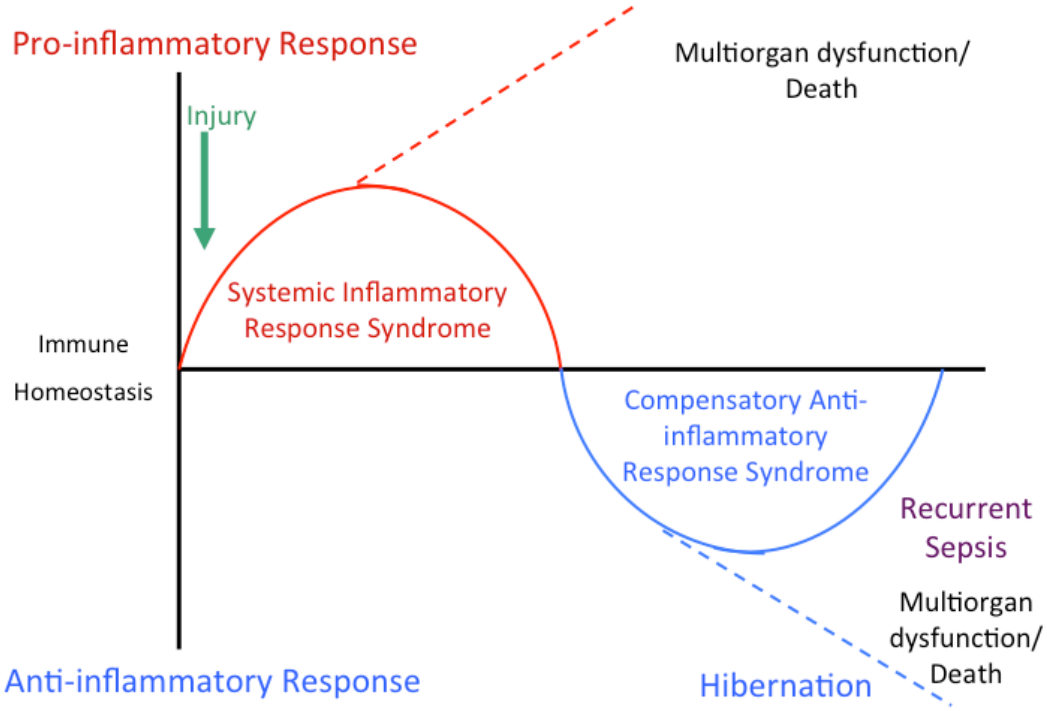
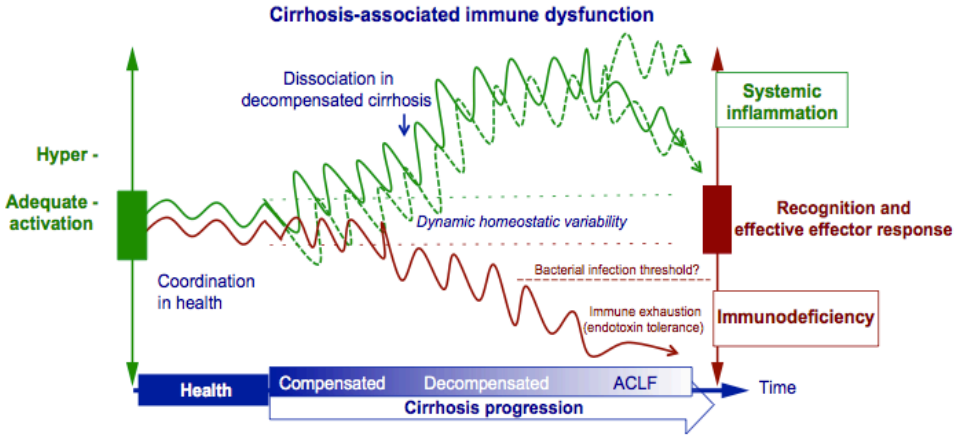


Figure 1.10 Systemic Inflammatory Response Syndrome (SIRS) and Compensatory Anti-Inflammatory Response Syndrome (CARS) dynamic

Interest in the presence of SIRS and potential imbalance of spontaneous pro-inflammatory response and reduced anti-inflammatory signaling pathway in cirrhotics has steadily grown (Figure 1.11) [110, 111] and this may be of even greater significance in AAH. In a study of 162 biopsy-proven AAH patients, in the presence or absence of infection, SIRS and LPS levels were major determinants of multi-organ failure and mortality [112]. The risk of exacerbating the underlying inflammatory state has been one of the main concerns in the development of immunotherapeutic approaches in AAH. Likewise anti-inflammatory therapies may render individuals progressively immunosuppressed and this difficult dynamic remains a challenge. The timing of when patients may move from SIRS into the CARS phase has not been extensively examined in AAH, and is also related to the timing of when individuals present to hospital which is highly variable.



Predominant CAID phenotypes

Immune characteristic	Pro-inflammatory	Immunodeficient
Pro-inflammatory cytokines (e.g., TNF-α, IL-6, IL-1β)	↑↑	↑
Anti-inflammatory cytokines (e.g., IL-10, TGFβ)	↑	↑↑
Phagocytosis (e.g., dendritic cells)	↑	↓
HLA-DR/co-stimulatory molecules expression on monocytes/macrophages	↑	↓
Expression of negative regulators (e.g., IRAK-M)	↓	↑

Figure 1.11 Cirrhosis-associated immune dysfunction (CAID)

Progressive liver disease is associated with pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)-driven stimulation. In compensated cirrhosis DAMPs released from damaged hepatocytes may initiate activation of the immune system and sterile systemic inflammation. In decompensated cirrhosis the increased translocation of bacterial products drives further activation of the immune system involving increased serum levels of pro-inflammatory cytokines. The predominantly pro-inflammatory CAID phenotype occurs in response to continuous PAMPs signalling and down-regulation of anti-inflammatory cytokines and negative feedback mechanisms. At end-stage cirrhosis the immune response is exhausted and the CAID phenotype switches to a predominantly 'immunodeficient' phenotype of impaired innate and adaptive protective immune responses [111].

1.5 Neutrophils

Neutrophils are a major innate immune cell subset involved in the first line of defense against infection. Conditions associated with neutropenia reveal that these cells are indispensible when it comes to control of bacterial and fungal infection. Rapidly recruited to sites of infection and inflammation, neutrophils engulf invading microbes via phagocytosis, then proceed to kill them by generating superoxide anions and hydrogen peroxide along with other ROS through activation of NADPH-oxidase, a process known as respiratory or oxidative burst.

Neutrophils were discovered more than 100 years ago, however knowledge of their function and, in particular, interaction with other cells of the immune system has expanded over the last few years. An overview of the role and function of this cell follows, including a summary of the current knowledge of their role in liver disease.

1.5.1 Neutrophils - role and function

1.5.1.1 Neutrophil life cycle

Neutrophils develop in the bone marrow from haematopoietic stem cells. Mature neutrophils are characterised by their segmented nucleus and granules that are filled with >700 proteins [113]. Granulocyte colony-stimulating factor (G-CSF) is a key factor regulating the neutrophil's life cycle by increasing cell proliferation, survival, differentiation, and trafficking/mobilization [114]. Neutrophils succumb via a number of possible death pathways (Figure 1.12).

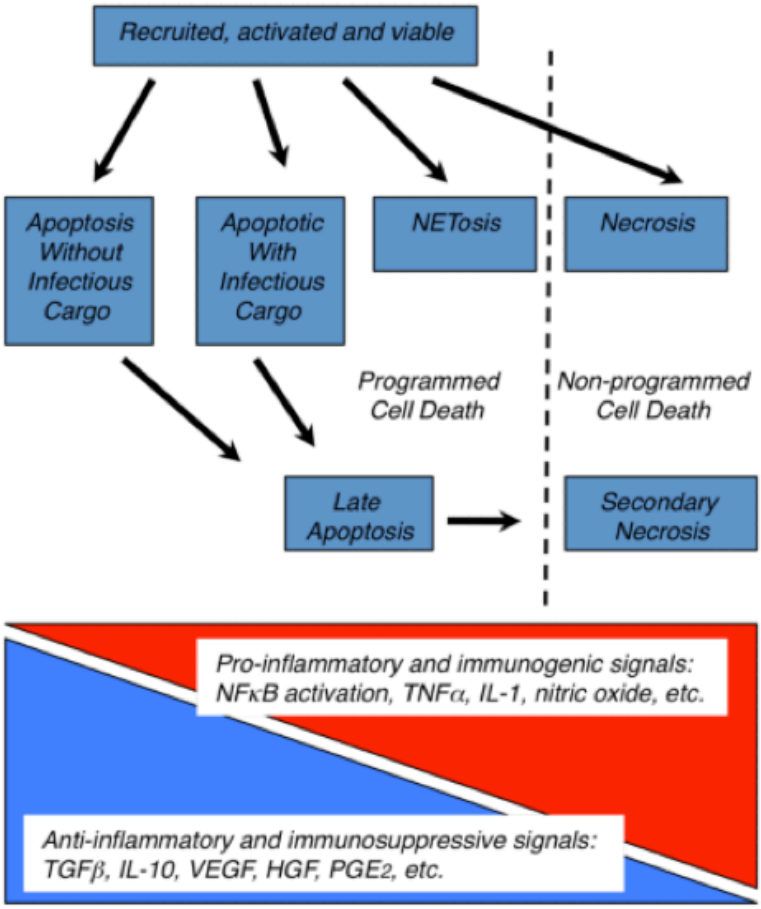


Figure 1.12 Possible death pathways for neutrophils and impact on resolution or perpetuation of inflammation [115].

Based on signaling for recognition by macrophages and the stage at which they are removed, inflammatory sequelae will vary from anti-inflammatory and immunosuppressive to pro-inflammatory and immunogenic.

Maintenance of neutrophil numbers is further regulated by clearance of apoptotic neutrophils by macrophages, a process termed "efferocytosis." Efferocytosis reduces the production of interleukin (IL)-23 and IL-17 and also dampens G-CSF production [116].

The lifespan of a neutrophil is generally measured in hours. The paradigm of neutrophils as short-lived immune cells has been challenged in recent years by *in-vivo* labelling studies, demonstrating a life span of up to 5 days for human neutrophils [117]. The methodology identifying this increased lifespan has,

however, been questioned as the technique almost certainly labelled bone marrow neutrophils. Nevertheless, during inflammation neutrophils become activated and their longevity is thought to increase by several fold [118]. This is clearly an important concept for studies examining neutrophil function in certain conditions; there is the possibility of skewing the analyses towards the active cells and excluding 'inactive' cells which may still have a role. As techniques of cell-labelling improve, knowledge on the differing age of circulating neutrophils and the potential clinical relevance of this will come to light.

In 2016 Potts *et al* conducted a pilot study examining intravascular granulocyte lifespan and showed that this was suppressed in compensated ARC compared to controls, this proved difficult to do in a small number of patients with AH and the authors state that with more refined techniques and increased patient numbers may enable measurement of granulocyte lifespan in other forms of chronic liver disease, including acute and acute-on-chronic liver failure [119].

The processes of neutrophil death and 'clear-up' are less well understood than recruitment. Enhanced understanding of neutrophil removal or its failure may allow opportunities to intervene to improve host defense or indeed prevent inappropriate injury.

1.5.1.2 The neutrophil recruitment cascade

The neutrophil recruitment cascade involves the following steps: tethering, rolling, adhesion, crawling and transmigration. The process is illustrated in figure 1.13 from Kolaczkowska and Kube's review in Nature Reviews Immunology [120].

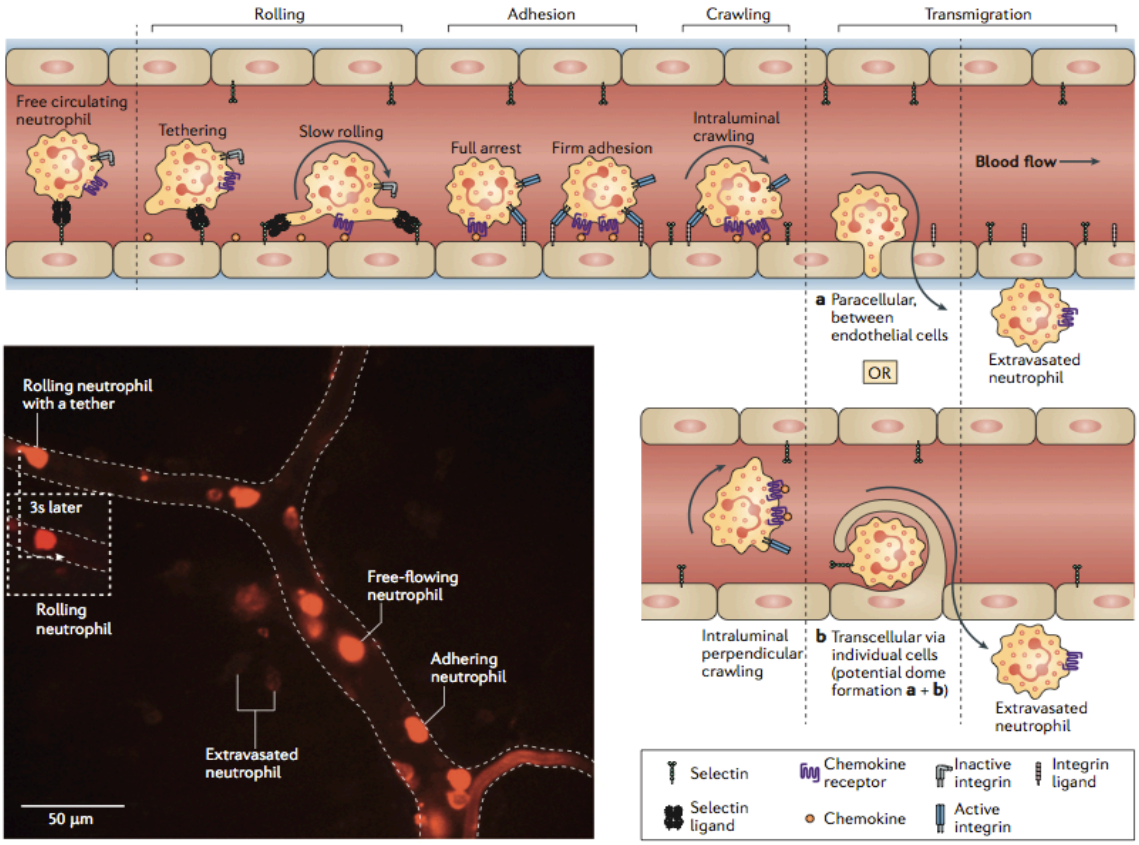


Figure 1.13 The updated neutrophil recruitment cascade from the vasculature to the tissue.

Two possible methods of transmigration are acknowledged: paracellular (between endothelial cells) a) and transcellular (through endothelial cells) b). Rolling is predominantly selectin-dependent, adhesion, crawling and transmigration depend on integrin interactions. The chemokine gradient guides crawling neutrophils along the endothelium. The intravital microscopy image shows a skin post-capillary venule with neutrophils (LY6G+ cells) labelled in red (phycoerythrin antibody conjugate), mouse skin was infected with *Staphylococcus aureus* and the image was taken 2 hours later. It captured neutrophils at different stages of migration [120].

Neutrophil recruitment is initiated by changes on the endothelium including up-regulation of selectins such as P-selectin and E-selectin which bind and activate receptors such as P-selectin glycoprotein ligand 1 (PSGL1) on the neutrophil cell surface [121]. Neutrophil receptors are discussed in 1.5.1.4.

1.5.1.3 Microbicidal mechanisms of neutrophils

Phagocytosis is the process by which certain cells engulf and destroy microorganisms and cellular debris. Generally these substances are coated with opsonins (antibodies and complement) to initiate binding with cell receptors on phagocytes – the first stage of phagocytosis. In the second stage the particle is

engulfed and enclosed forming a phagosome. The third stage involves the merging of phagosomes and lysosomes with the formation of oxygen radicals (respiratory or oxidative burst) which ultimately results in the killing of the engulfed bacteria.

In the 1950s myeloperoxidase, known to be released from granules during phagocytosis, was found to be involved in generating hydrogen peroxide, then further experimentation clarified that another oxidase, NADPH oxidase, also played an important role in the generation of hydrogen peroxide and ROS [122]. It is now known that neutrophils have other microbicidal mechanisms such as defensins and broadly acting proteases, however phagocytosis with generation of ROS and hypochlorous acid is still regarded as the critical killing mechanism for most invading pathogens [123, 124].

Neutrophil granules are reservoirs of proteins that can kill microbes and digest tissue. They are classified into three distinct subsets based on the presence of characteristic granule proteins: primary (azurophil) granules (myeloperoxidase, MPO), secondary (specific) granules (lactoferrin), and tertiary (gelatinase granules) [125].

Lactoferrin can bind and transport iron but is thought to have not only antibacterial but antiviral and antiparasitic properties [126]. Lactoferrin possesses a high affinity binding motif to the lipid A portion of LPS [127]. It's direct antimicrobial activity in animal models provided a basis for exploring it's role in humans as a treatment; orally administered human recombinant lactoferrin was investigated in a phase 2 trial in adult patients with severe sepsis,

the initial positive results however were not borne out in a follow up phase 2/3 trial [127, 128].

1.5.1.4 Neutrophil cell surface receptors

The function of the neutrophil is mediated by a number of cell surface receptors. Intracellular signal transduction pathways are then activated, which are complex and not completely understood. There are several classes of receptors expressed on the surface of neutrophils, including G-protein-coupled seven-transmembrane receptors, Fc-receptors, adhesion molecules like selectins/selectin ligands and integrins, various cytokine receptors, as well as innate immune receptors including Toll-like receptors (TLRs) and C-type lectins (Table 1.1) [129].

Table 1-1 Neutrophil receptors [129]

G-protein-coupled receptors	Fc-receptors	Adhesion receptors	Cytokine receptors	Innate immune receptors
Formyl-peptide receptors FPR1(FPR) FPR2(FPRL1) FPR3(FPRL2) ***Classical chemoattractant receptors*** BLT1(LTB_4-rec.) BLT2(LTB_4-rec.) PAFR C5aR ***Chemokine receptors*** CXCR1(human) CXCR2 CCR1 CCR2	***Fcγ-receptors*** FcγRI FcγRIIA(human) FcγRIIB(inhibitory) FcγRIII(mouse) FcγRIIIB(human) FcγRIV(mouse) ***Fcα-receptors*** FcαRI (human) ***Fcε-receptors*** FcεRI FcεRII	***Selectins and selectin ligands*** L-selectin PSGL-1 ***Integrins*** LFA-1($\alpha_L\beta_2$) Mac-1($\alpha_M\beta_2$) VLA-4($\alpha_4\beta_1$)	***Type I cytokine receptors*** IL-4R IL-6R IL-12R IL-15R G-CSFR GM-CSFR ***Type II cytokine receptors*** IFNAR(IFNα/βrec) IFNGR IL-10R ***IL-1R family*** IL-1RI IL1RII(decoy) IL-18R ***TNFR family*** TNFR1(p55) TNFR2(p75) Fas LTβR RANK TRAIL-R2 TRAIL-R3	***Toll-like receptors*** TLR1 TLR2 TLR4 TLR5 TLR6 TLR7(?) TLR8 TLR9 ***C-type lectins*** Dectin-1 Mincle MDL-1 Mcl CLEC-2 ***NOD-like receptors*** NOD2 NLRP3 ***RIG-like receptors*** RIG-I MDA5

A number of these neutrophil receptors will be discussed in more detail.

1. G-protein coupled receptors

G-protein coupled receptors include formyl-peptide receptors which sense bacterial products and tissue injury, receptors for a range of chemoattractants and chemokine receptors. These receptors activate the chemotactic migration of neutrophils, binding of these receptors also triggers ROS production and 'primes' the cell resulting in an augmented response to subsequent stimulation from other agonists.

2. Fc-receptors

Neutrophils express Fc-receptors, these are primarily involved in recognition of Ig-opsonised pathogens but also play a role in immune-complex mediated inflammatory processes. The most important Fc-receptors in neutrophils are the low affinity Fcγ-receptors [130]. Human neutrophils express FcγRIIA (CD32), a single-chain transmembrane receptor which carries an immunoreceptor tyrosine-based activation (ITAM) in its cytoplasmic tail and FcγRIIIB (CD16b), an extracellular molecule anchored to the plasma membrane by a GPI moiety [129]. Low affinity, particularly FcγRIIIB, play important roles in immune-complex mediated activation of neutrophils [131].

3. Adhesion receptors

The two main groups of adhesion receptors are the selectins/selectin ligands and integrins. Selectins are transmembrane glycoproteins and mediate interactions between leukocytes and the vessel wall. The most important integrins expressed on leukocytes belong to the β2 integrin family [132].

4. Cytokine receptors

The cytokine receptors are divided into 4 main subsets as outlined in table 1.1.

5. Innate immune receptors

Toll-like receptors are innate immune receptors involved in the direct recognition of pathogens or tissue damage (Figure 1.14). Neutrophil TLRs recognise various microbial structures leading to increased cytokine and chemokine production, priming and delayed apoptosis of the cells [129].

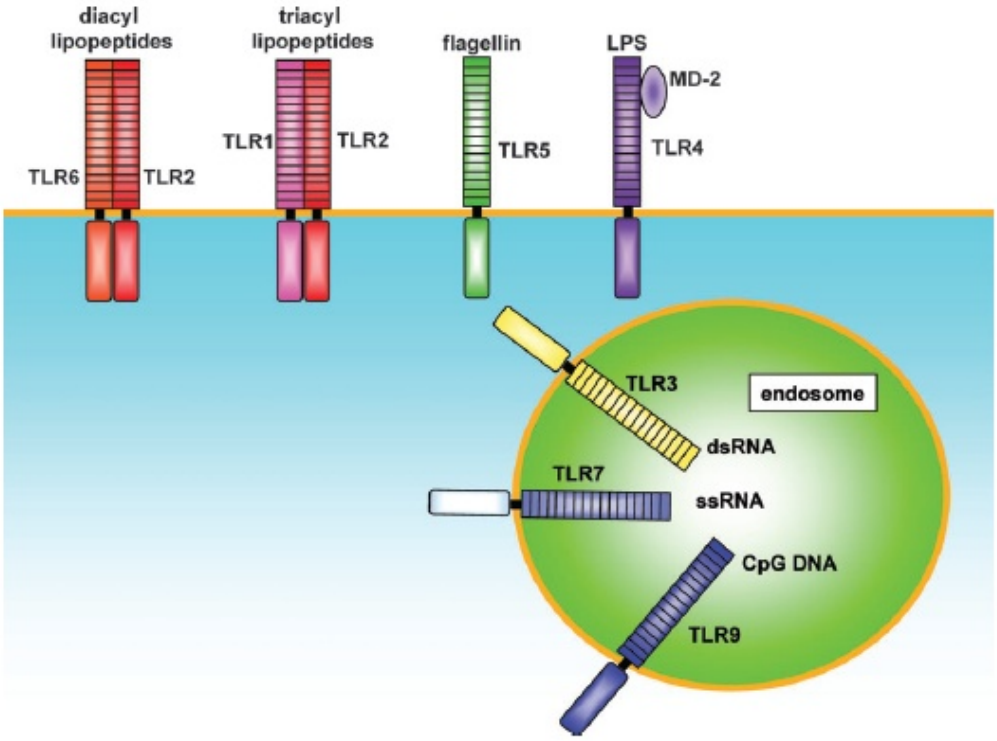


Figure 1.14 Toll like receptors [133]

Neutrophils express all TLRs except TLR3. The TLRs have multiple ligands, however TLR4 has been predominantly associated with the recognition of LPS, a component of the outer membrane of gram-negative bacteria and TLR2 the peptidoglycans of gram-positive bacteria.

TLR4 forms a complex with other proteins for ligand recognition. CD14 is a glycosylphosphatidylinositol-anchored membrane protein (and also exists in a soluble form) which binds LPS attached to LPS binding protein (LBP), this then associates with TLR4 to form a functional LPS receptor complex [134]. Binding of LPS is also thought to require the MD2 protein [135]. Further study suggests that CD14 and LBP only enhance TLR4-LPS binding and signalling and are not absolutely required for this process [136].

1.5.1.5 Neutrophil extracellular traps

Neutrophil extracellular traps (NETs), first described in 2004, are extracellular strands of DNA bound to antimicrobial neutrophil-derived peptides and proteins [137]. NETs are web-like structures thought to trap pathogens and their formation is also thought to be an alternative to death by apoptosis or necrosis [125]. NET proteins are primarily the cationic bactericidal proteins: histones, defensins, elastase, proteinase 3, heparin binding protein, cathepsin G, lactoferrin and MPO [138]. NETs seem to contribute to autoimmune diseases, in which the target antigens are frequently the constituents of NETs (for example MPO/proteinase 3) and intravascular NETs may damage the endothelium further exacerbating capillary leak in sepsis and acute lung injury [139]. The full contribution of NETs to overall antimicrobial defense has not yet been established and strategies for the elimination of NETs in non-infectious conditions may be beneficial. Recently there has been some interest in NET formation and potential contribution in liver disease.

1.5.2 Neutrophils - role in liver disease

As outlined above, neutrophils are vital for host defense and removal of cell debris but can also cause associated, bystander tissue damage. Neutrophils play a critical role in liver disease and evidence has accumulated in relation to several liver diseases including hepatic ischaemia/reperfusion injury, viral hepatitis, non-alcoholic fatty liver disease, ALD, cirrhosis, liver failure and HCC [140].

Neutrophil-mediated parenchymal cell damage in the liver is thought to be initiated by the priming and subsequent accumulation of neutrophils in the hepatic sinusoids (Figure 1.15) [141].

The initial step is triggered by pro-inflammatory mediators, and after receiving a chemotactic signal neutrophils can extravasate and attack the target cell, the hepatocyte. Generation of ROS, especially hypochlorous acid, as described above triggers an intracellular oxidant stress in the target cell and causes cell death [142].

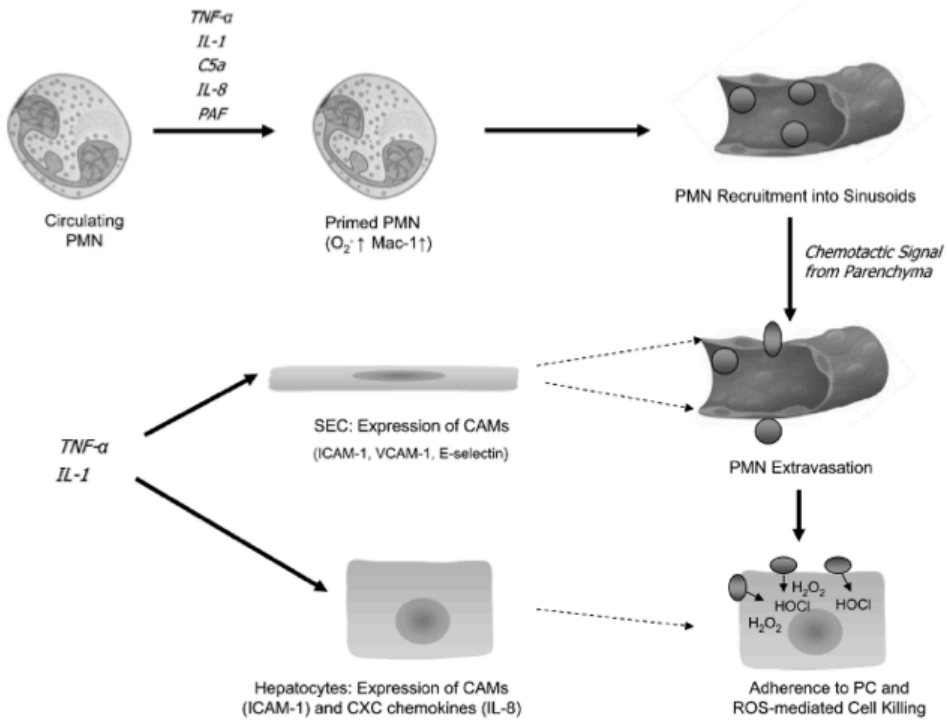


Figure 1.15 Proposed mechanisms of neutrophil mediated liver injury from Jaeschke and Hasegawa's review [141]

The migration of neutrophils through the endothelium and into the liver parenchyma is essential for alcohol-induced hepatic inflammation [140]. This process is dependent on neutrophil integrin receptors and the endothelial surface. The expression of hepatic E-selectin has been found to be very important for neutrophil infiltration into the liver and in the pathogenesis of the early stages of ALD [87]. The initial innate response that leads to AAH may be triggered by alcohol in the liver and the increased translocation of LPS which

activates Kupffer cells. As discussed in 1.3 it is believed that activated Kupffer cells produce a variety of cytokines and chemokines, including IL-8, RANTES, IL-17 that subsequently recruit neutrophils to the liver [68]. Serum levels and liver expression of CCL2 are increased in AAH and were found to be associated with disease severity; CCL2 liver expression correlated with neutrophil infiltrate and IL-8 expression [143].

Recently there has been interest in NETs (described above) and whether these may mediate bacterial induced liver damage. Kolaczkowska *et al* demonstrated that after MRSA infection, bacteria accumulated in the liver more readily than any other organ as they were captured by Kupffer cells in the liver. Subsequently recruited neutrophils released NETs and these were found to cause the majority of liver injury seen, rather than the bacteria itself [144]. This may in fact be of relevance in the development of AAH and warrants further exploration.

1.5.3 Neutrophils – defects in liver disease

Neutrophil dysfunction both in acute and chronic liver disease has long been recognised [145]. The most commonly reported defect in cirrhosis is the impaired phagocytosis of opsonized bacteria [93, 146-148]. There is also defective oxidative burst, myeloperoxidase activity and a lower response to the peptidoglycan recognition protein with resultant impaired microbicidal activity [93, 149, 150]. Impaired chemotaxis is seen, through reduced adhesion to endothelial cells and decreased transendothelial migration [147, 151]. In Taylor *et al's* study of neutrophil function in cirrhosis across all aetiology, baseline circulating neutrophil dysfunction was found to be a predictor of outcome and 90-day and 1-year survival [152]. Cirrhotic patients who had increased basal

production of ROS appeared to be at the highest risk of developing complications and multi-organ dysfunction. Neutrophil dysfunction has been linked to persistent *in vivo* stimulation, especially observed in patients with higher serum levels of pro-inflammatory cytokines [147] and ammonia [153]. It is perhaps this persistent *in vivo* stimulation, resulting in increased basal ROS, which renders the cells unable to appropriately phagocytose with reduced stimulated burst on pathogen exposure, this is discussed further in 1.8.

In severe AAH blood neutrophils are activated as shown by increased H_2O_2 production and decreased L-selectin expression, TNF-α and IL-8 plasma and tissue levels were found to be markedly increased and IL-10 reduced, during steroid therapy a normalization of these parameters was observed [61]. A control group of AAH patients without corticosteroid therapy were not evaluated in this study.

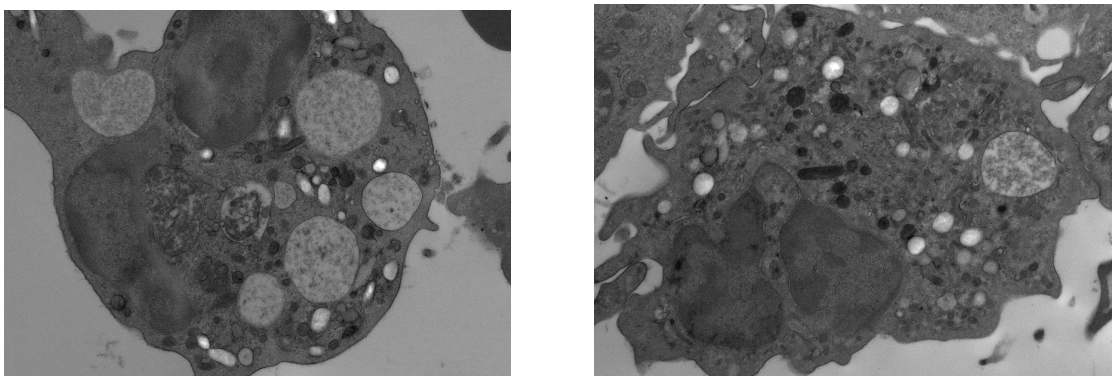


Figure 1.16 Figure demonstrating transmission electron microscopy (x4800) pictures of a healthy control neutrophil (left) and a neutrophil from a patient with severe acute alcoholic hepatitis which shows enlarged nuclei and dense cytoplasm.

Manakkat Vijay, Ryan and Shawcross (unpublished data).

Mookerjee *et al* found that a resting burst ≥ 55% and a reduced phagocytic capacity ≤ 42% was associated with significantly greater risk of infection, organ failure and mortality [154]. The *ex vivo* removal of endotoxin in this study resulted in improved neutrophil function. Stadlbauer *et al* went on to investigate

neutrophil TLRs in mediating neutrophil dysfunction in AAH [155]. Neutrophils from healthy volunteers were incubated with alcoholic hepatitis patients' plasma (n=12) with and without TLR2, 4 and 9 antagonists and with and without albumin. Patients' plasma increased oxidative burst, decreased CXCR1 and 2 expression, decreased phagocytosis of normal neutrophils and increased expression of TLR2, 4, and 9. Inhibition of TLRs prevented the increase in burst and decrease in CXCR1 and 2 expression but did not prevent phagocytic dysfunction. Incubation with albumin prevented the plasma induced neutrophil dysfunction [155].

1.5.4 HL-60 cell line

Neutrophils are considered to have a short life span (6-12 hours) and this has to be taken into consideration when performing experiments. It is possible to isolate neutrophils from whole blood using commercial gradients (Polymorphprep, AxisShield). There are, however, some concerns when using primary cells for certain experiments. There is natural heterogeneity even within one person [156]. Neutrophils are easily activated, experiments need to be performed quickly after blood is drawn and they can, therefore, be difficult to use in culture.

HL-60 cells were first isolated in 1977 from a 36-year old woman with acute promyelocytic leukaemia [157]. The cells were kept in continuous culture and deposited after 8 passages at the American Tissue Culture Central (ATCC), they are distributed at passage 21. The original reason for studying these and other myeloid cells was to improve understanding and treatment for leukaemia [158]. It was then discovered that these cells, under certain conditions, differentiate

toward a neutrophil-like state and the study of these cells has provided useful insight into neutrophil biology.

HL-60 cells can be induced towards monocytes, eosinophil or granulocyte-like cells (for a review see Collins [159]). The most common ways to differentiate cells towards a granulocytic lineage is with either dimethylsulphoxide (DMSO) or all-trans-retinoic acid (ATRA) [159]. Upon differentiation the cells become smaller and there is induction of cell surface markers [160], however they fail to develop specific granules [161]. These cells have been used to further examine phagocytosis and microbicidal activity of neutrophils [162-164].

1.6 Interferons

Interferon (IFN) was first described in 1957 as a cell product capable of interfering with viral replication in cells infected with homologous or heterologous viruses [165]. IFNs are now recognized as central regulatory mediators of the immune response with three major biological activities: antiviral, anti-tumour and immunoregulatory activity [166].

IFNs have been classified into three types. Type I IFNs include 13 forms of IFN-α, one form of IFN-β, one form of IFN-ω, one form of IFN-ε, and one form of IFN-κ [167]. These molecules signal through a ubiquitously expressed receptor composed of two chains: IFN-αR1 and IFN-αR2. Only one type II IFN has been identified: IFN-γ, this was originally described in 1965 as a virus inhibitory protein [168]. IFN-γ signals through a ubiquitously expressed receptor composed of the IFN-γR1 and IFN-γR2 subunits [167].

The type III interferons, IFN-λ1, -λ2 and -λ3 (or IL-29, IL-28A and IL28B), were discovered in 2003 [169, 170]. Type III IFNs act through a receptor composed of two chains, an IFN-λ-specific IFN-λR1 expressed on certain cell types and a ubiquitously expressed IL-10Rβ, which also forms part of the receptors for IL-10, IL-22 and IL-26 [171]. In addition to the antiviral properties of IFN-λ there is increasing evidence that this class of cytokine also plays a significant role in the modulation of adaptive immunity, autoimmunity and tumour progression [172].

1.6.1 Interferons – role in alcohol-related liver disease

The most studied type of IFN in this area has been IFN-α in individuals with hepatitis C virus (HCV) and excess alcohol consumption. Ethanol metabolites impair IFN-α signalling through various pathways providing the mechanism for enhanced HCV-infection severity by excess alcohol consumption in HCV-infected individuals [173].

Serum levels of IFN-γ have been shown to be elevated in chronic liver disease. When compared to levels of IL-1β, TNF-α and IL-6, serum levels of IFN-γ were however increased in a smaller proportion of patients and did not further increase with progression to cirrhosis [174]. In ALD the number of CD8+ and CD4+ T lymphocytes in the liver increases [72]. Steatohepatitis is associated with a T helper (Th) 1 cytokine response characterised by IFN-γ and TNF-α elevation [175]. When T cells were further examined in AAH the IL-17 pathway was found to be activated, IFN-γ was found to be of less significance in this study [176].

The role of IFN-λ in ALD has not been examined.

1.6.2 Interferon lambda

IFN-λ has a restricted cell-response pattern and is thought to be of specific importance in antiviral protection [177, 178]. There has been particular interest in the role of IFN-λ in HCV and hepatitis B infections, and genome-wide association studies have identified IFN-λ polymorphisms that are associated with improved outcome both in terms of spontaneous clearance and response to treatment [178, 179]. The role of IFN-λ in bacterial infection is not known. IFN-λ is thought to be of particular importance at epithelial surfaces (Figure 1.17), as above, the most studied biological role of this class of cytokine has been their antiviral activity. Epithelial surfaces experience constant microbial exposure and it is thought that IFN-λ signaling could control low-level infections without broadly activating a systemic pro-inflammatory response [172]. Given the role of bacterial translocation in the pathogenesis of ALD, this may be of significance.

Whilst a number of different cell types have been reported to produce IFN-λ in response to viral infection, including monocytes, monocyte-derived dendritic cells [180, 181] and plasmacytoid dendritic cells [182] little is understood regarding the production and source of IFN-λ in bacterial infection and in inflammatory conditions such as ALD. Recently, Blazek *et al.* have demonstrated that IFN-λ2 targets neutrophil chemotaxis in a mouse model of inflammatory arthritis [183] suggesting a relationship between IFN-λ and neutrophils. The interplay between IFN-λ and neutrophils in healthy and other disease states has not been explored and the question as to whether neutrophils produce IFN-λ is unanswered.

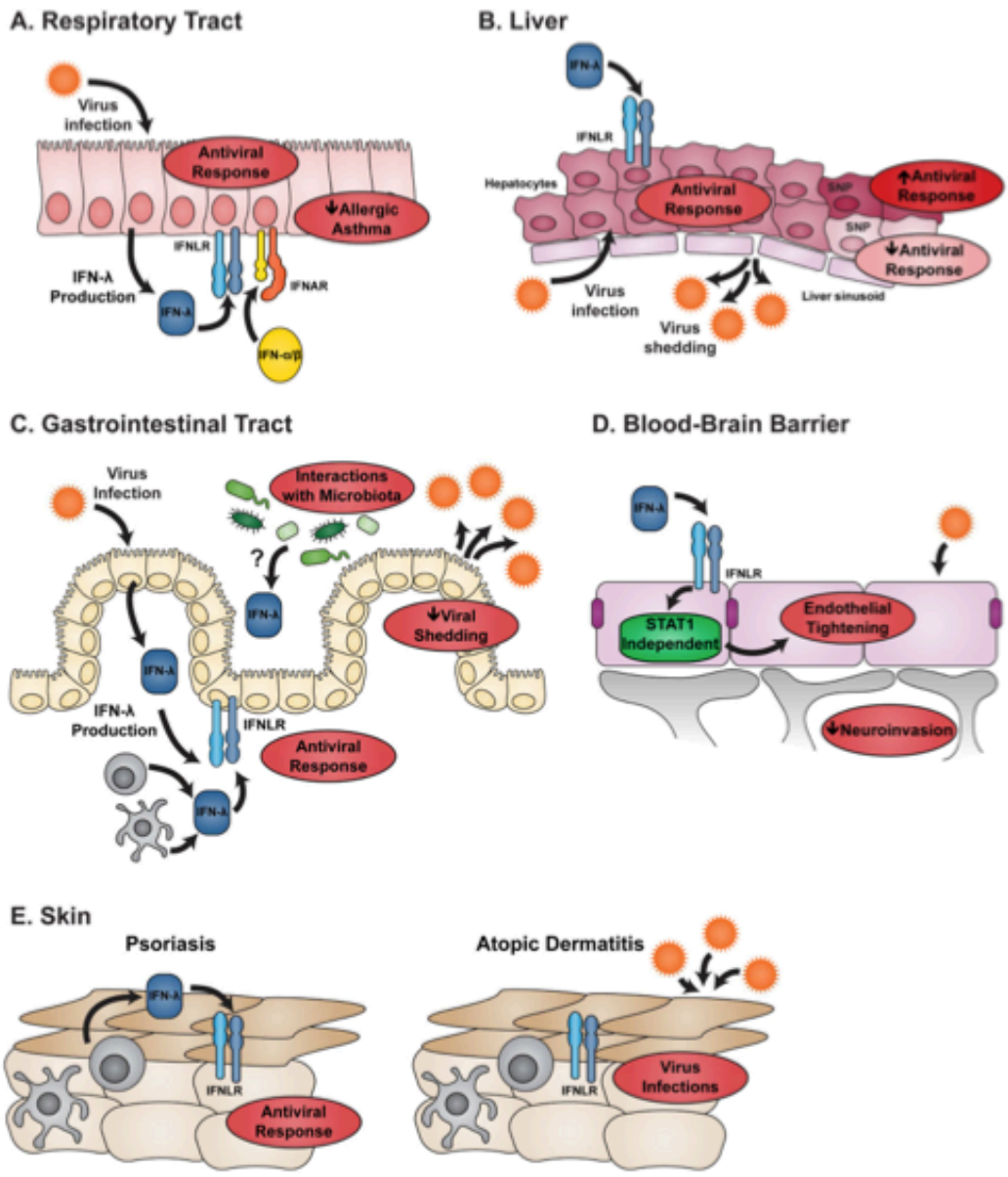


Figure 1.17 Antiviral effects of IFN-λ at barrier surfaces [172]

Hepatocytes express the IFN-λ receptor and are highly responsive to IFN-λ (B). Immunity in the gastrointestinal tract is shaped by the microbiome. The interactions between the virome and microbiome remain unclear and it is thought the ability of gut microbes to promote viral persistence requires IFN-λ-signalling (C). IFN-λ-signalling is also of importance within the respiratory tract, brain and skin (A, D, E).

1.7 Management of alcohol-related liver disease

1.7.1 Supportive measures

The management of ARC focuses on alcohol abstinence, aggressive nutritional therapy and prophylaxis of cirrhosis complications [10]. The importance of sepsis has been discussed in 1.4.

Referral to alcohol misuse specialists should be made at the earliest opportunity and the involvement of addiction specialists should be sought. Ongoing alcohol use after diagnosis is the most important factor increasing the risk of complications and death [46, 47, 184]. Monitoring and treatment for alcohol withdrawal in the acute setting is required. Figure 1.18 outlines the broad recommendations in the management of ARC.

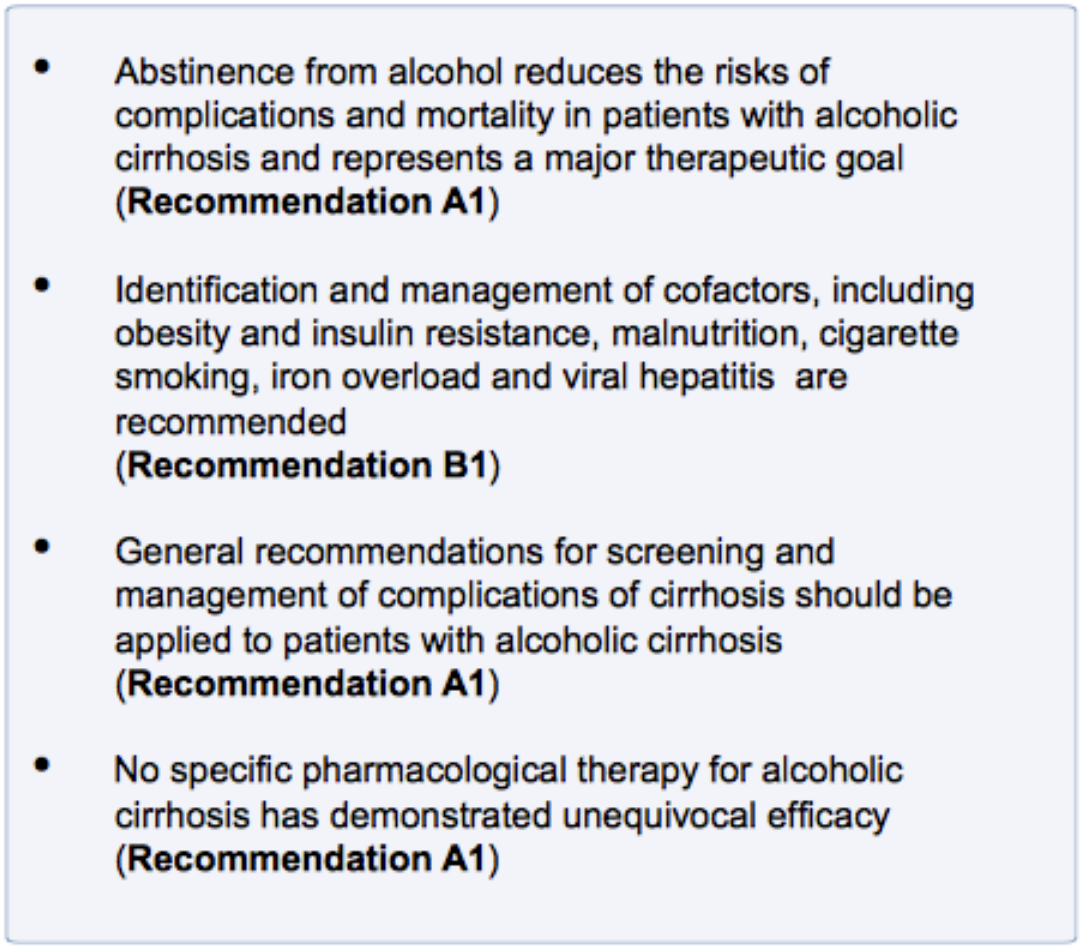


Figure 1.18 Recommendations on the management of ARC from the EASL clinical practice guidelines [10]

Onset of decompensation in ALD should prompt clinicians to suspect superimposed AAH.

1.7.2 Liver transplantation

Most liver transplant programmes recommend a 6-month period of abstinence prior to liver transplantation assessment. This is presumed to serve two purposes, first, to allow some individuals to recompensate and avoid the need for liver transplant and second, to identify patients likely to maintain abstinence. The latter point has come under scrutiny particularly in recent years and the data pertaining to the 6-month rule as a predictor of long-term abstinence are controversial [185, 186]. Liver transplantation confers a survival benefit in

patients with ARC classified as Child-Pugh C or MELD≥15 [10]. ALD does not influence liver transplant survival benefit [187].

A significant proportion of patients with AAH fail to respond to medical therapy and abstinence. In 2011 Mathurin *et al.* [100] published a prospective study of liver transplantation in 26 patients who failed to respond to 1 week of prednisolone therapy. Of those transplanted 77% survived for 6 months, similar to a matched group of patients who responded to prednisolone and significantly better than a matched group of patients with AAH and a Lille score greater than 0.45 who did not undergo liver transplantation. Two-year survival in the transplanted group was 71%. Less than 2% of patients with AAH received a liver transplant and a minor proportion of livers were used for transplantation into patients with AAH. A larger study is underway in France. The UK pilot study to permit transplantation for highly select patients with AAH ran for several years, but there were no patients transplanted and the study is currently suspended.

1.7.3 Specific therapies in severe alcoholic hepatitis

Recent onset jaundice helps to distinguish patients with AAH from those with decompensated cirrhosis. Diagnosis and scoring systems used are described in 1.1 and 1.2. A management algorithm for AAH is outlined in figure 1.19.

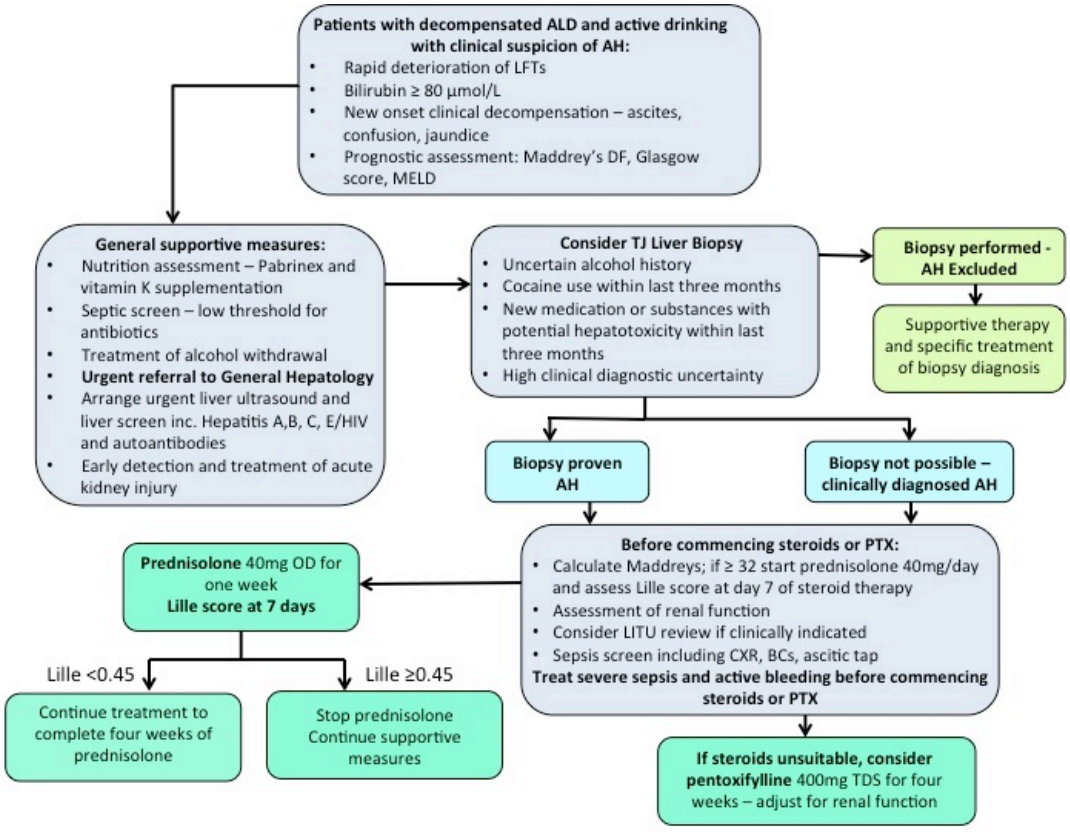


Figure 1.19 Therapeutic algorithm for suspected AAH.

A Lille score at seven days of ≥0.45 indicates non-response to steroids and increased risk of infection and death. Figure adapted from EASL Clinical Practical Guidelines: Management of Alcoholic Liver Disease. Blackmore, Ryan, Kings College Hospital Alcoholic Hepatitis Management Guidelines

Corticosteroids

From 1971 to 2014 there were 13 randomised trials and 4 meta-analyses that investigated the use of corticosteroids in AAH [188-191]. There have been disparate results and their role in this condition remains contentious. Concerns regarding their use have centered around the risk of sepsis and gastrointestinal haemorrhage. The largest placebo controlled study of steroids in AAH was performed in 90 patients and found prednisolone to provide no benefit compared to placebo [38]. In studies where histological confirmation of the diagnosis was required prednisolone was associated with a short-term reduction in mortality [192, 193]. The Cochrane meta-analysis reported only a trend towards increase in survival [189]. A re-analysis of the 3 largest trials indicated

that steroids significantly increased the short-term survival of patients with AAH [190]. The Steroids or Pentoxifylline for Alcoholic Hepatitis [STOPAH] trial was conducted between 2011 and 2014 (during the time that much of my laboratory-based research was conducted) in an attempt to resolve the controversy regarding the use of steroids or pentoxifylline. The trial is the largest study to date in AAH. This was a double-blind, factorial 2 x 2, multicentre trial conducted in the UK between 2011 and 2014 [98]. 1103 patients with a clinical diagnosis of AAH were randomly assigned to one of four groups; one receiving pentoxifylline-matched placebo and prednisolone-matched placebo, a second group receiving 40mg of prednisolone daily and a pentoxifylline-matched placebo, the third group receiving 400mg of pentoxifylline three times daily and prednisolone-matched placebo, and the fourth group receiving 40mg of prednisolone daily and 400mg of pentoxifylline three times daily. This study reported a reduction in mortality at 28 days for patients given prednisolone compared with control patients but this did not reach significance and survival curves converged after 28 days. Incident infections were more common among subjects given prednisolone than controls.

Nutrition

Protein calorie malnutrition is common in severe AAH and is associated with impaired survival [194]. Nasogastric feeding can be difficult to administer however, with impaired tolerance, particularly among patients with hepatic encephalopathy. A meta-analysis of 9 trials of enteral feeding and 4 trials of parenteral nutrition found that these strategies produce a modest reduction in mortality [195]. In a recent randomized trial of patients with severe AAH treated with steroids, intensive enteral nutrition was difficult to implement and did not

increase survival [196]. The authors did conclude that low daily energy intake was associated with greater mortality, so adequate nutritional intake should be a main goal of treatment. Although there is room for further high quality trials, assessment of nutritional status and provision of appropriate supplementation should form part of standard supportive care in all patients with AAH.

Pentoxifylline

Pentoxifylline, a phosphodiesterase inhibitor, is thought to inhibit production of TNF. Akrividias *et al.* reported that 24.5% (12/49) of AAH patients given pentoxifylline died within 6 months, compared to 46.1% (24/52) given placebo, the authors state that the benefit appeared to be related to a significant decrease in the risk of developing hepatorenal syndrome [197]. A meta-analysis of five trials that compared pentoxifylline to placebo concluded that no conclusions could be drawn regarding whether pentoxifylline had a positive, negative or neutral effect on patients with AAH [198]. Four of the five trials were deemed to have a high risk of bias. In a study of placebo vs pentoxifylline in 335 patients with Child Pugh C cirrhosis (255 ALD, with 133 AAH), there was no significant difference in short-term mortality between those given pentoxifylline vs placebo in the overall study and in those with AAH [199]. Pentoxifylline was, however, shown to reduce the risk of complications including bacterial infection, renal insufficiency, hepatic encephalopathy and gastrointestinal haemorrhage. The combination of pentoxifylline and prednisolone was evaluated in 4 studies; results did not show that the combination was superior [15]. Mathurin and colleague's study that assigned 270 patients to a combination of pentoxifylline and prednisolone or prednisolone alone did not demonstrate any difference in survival. The study may have been underpowered to detect a significant

difference in HRS which was lower in those receiving both drugs [200]. In the STOPAH study, pentoxifylline did not improve outcomes, and there was no statistically significant difference in acute kidney injury between those that received pentoxifylline and those who did not [98].

Inhibitors of TNF

Animal work and two small pilot studies indicated that there might be therapeutic potential of utilising monoclonal antibodies against TNF in AAH [201].

Naveau *et al.* went on to conduct a double-blind, randomized study of prednisolone vs the combination of prednisolone and infliximab (10mg/kg at week 0, week 2, and week 4) in 36 patients with severe AAH [202]. Increased infection and mortality was seen in the infliximab arm and the study was therefore stopped early. Similar problems were found in a trial of etanercept; infection rates and 6-month mortality was higher in the etanercept group compared to the placebo group [203].

N-Acetylcysteine

Oxidative stress is thought to be one of the key mediators in liver injury in AAH. The levels of antioxidants are decreased in liver disease and several studies, therefore, have tested the safety and efficacy of N-acetylcysteine (NAC) alone or in combination with other antioxidants (Figure 1.20).

Placebo and standard of care	NAC and standard of care	NAC, with or without antioxidants	Prednisolone	Prednisolone and NAC	Study
12.5% (3/24)	32.1% (9/28)	NA	NA	NA	Moreno et al[38a]
38% (n = 34)	35% (n = 36)	NA	NA	NA	Stewart et al[39b]
NA	NA	45.8% (22/48)	30.2% (16/53)	NA	Philipps et al[40]
NA	NA	NA	22.5% (20/89)	8.2% (7/85)	Nguyen-Khac et al[7]

NA, not applicable.
[a]Standard of care comprised enteral nutritional supplementation with oral supplementation with B vitamins, folic acid phosphorus, zinc, and magnesium. The NAC group received intravenous NAC for 14 days, and the placebo group received 5% dextrose for 14 days.
[b]Standard of care included prednisolone if the patient was not infected or had recent upper gastrointestinal bleeding. Approximately 56% of patients received prednisolone. Patients received intravenous NAC for 1 week and oral antioxidants (such as vitamins A–E, selenium, zinc, folic acid, or coenzyme Q, and so forth) daily for 6 months. Mortality values were determined after 60 days, based on Kaplan–Meier survival analyses.

Figure 1.20 One-month mortality of patients with alcoholic hepatitis given N-acetylcysteine and/or antioxidants alone or in combination with standard of care [15]

The study with the most promising results was reported by Nguyen-Khac *et al.*; the combination of NAC and prednisolone increased 1-month survival, reduced infections and HRS compared with prednisolone alone [204]. Mortality was lower at 6-months (the primary outcome) but this was not significant. This strategy warrants further investigation.

Granulocyte Colony-Stimulating Factor

In 2014 Singh *et al.* published their study on the effects of pentoxifylline vs a combination of pentoxifylline and granulocyte colony-stimulating factor (G-CSF) in AAH [205]. The aim of the study was to test the hypothesis that G-CSF might mobilise bone marrow derived stem cells and promote hepatic regeneration and survival. A significantly larger proportion of patients who received pentoxifylline with G-CSF survived for 90 days (18/23) than those who received only pentoxifylline (5/23). Fewer patients receiving combination therapy died of infection and liver failure. G-CSF is a potent stimulus of neutrophil function and it is not clear whether this is the main mechanism by which therapy improved survival or whether this relates to improved liver regeneration as was hypothesized. Further studies are required, in particular evaluation of G-CSF in combination with prednisolone.

A recent network meta-analysis compared outcomes among multiple treatments, based on 22 studies deemed high quality comprising more than 2500 patients with AAH [191]. The authors concluded that pentoxifylline alone, corticosteroid alone, corticosteroid + NAC and corticosteroid + pentoxifylline are superior to placebo in decreasing short-term mortality. These interventions were found to be generally comparable to each other, though the addition of NAC to corticosteroid may infer additional benefit. This was a well-performed meta-analysis however several points should be kept in mind; 40% of the patients included were supplied by the STOPAH study. There were also considerable differences in one-month survival among trials and only 1 study has examined the effect of the addition of NAC to prednisolone compared to prednisolone alone. In future it may be possible to carefully tailor treatments to individuals, including true discernment at presentation of those who are likely to benefit from certain agents pre-exposure.

A finding that was reinforced in the network meta-analysis was that there are no agents that decrease medium or long-term mortality in patients with severe AAH. Importantly, the report concludes that a combined pharmacological and psychotherapeutic approach to treating these patients is warranted in order to improve long-term mortality.

1.7.4 Potential new treatments

Figure 1.21 outlines potential therapeutic targets and strategies. Antibiotics and probiotics to alter the gut microbiome are being evaluated as are LPS-binding antibodies. There is a careful balance to be struck, as evidenced by the anti-TNF trials, in obtaining control of inflammation yet avoiding life-threatening immune

suppression. A meta-analysis of placebo controlled trials did not confirm the difference seen in infection rates between those exposed to steroid therapy and those not. Nevertheless the concern of heightened susceptibility to infection in this condition whilst on steroid therapy has formed the basis for several new studies combining steroid and prophylactic antibiotics [15].

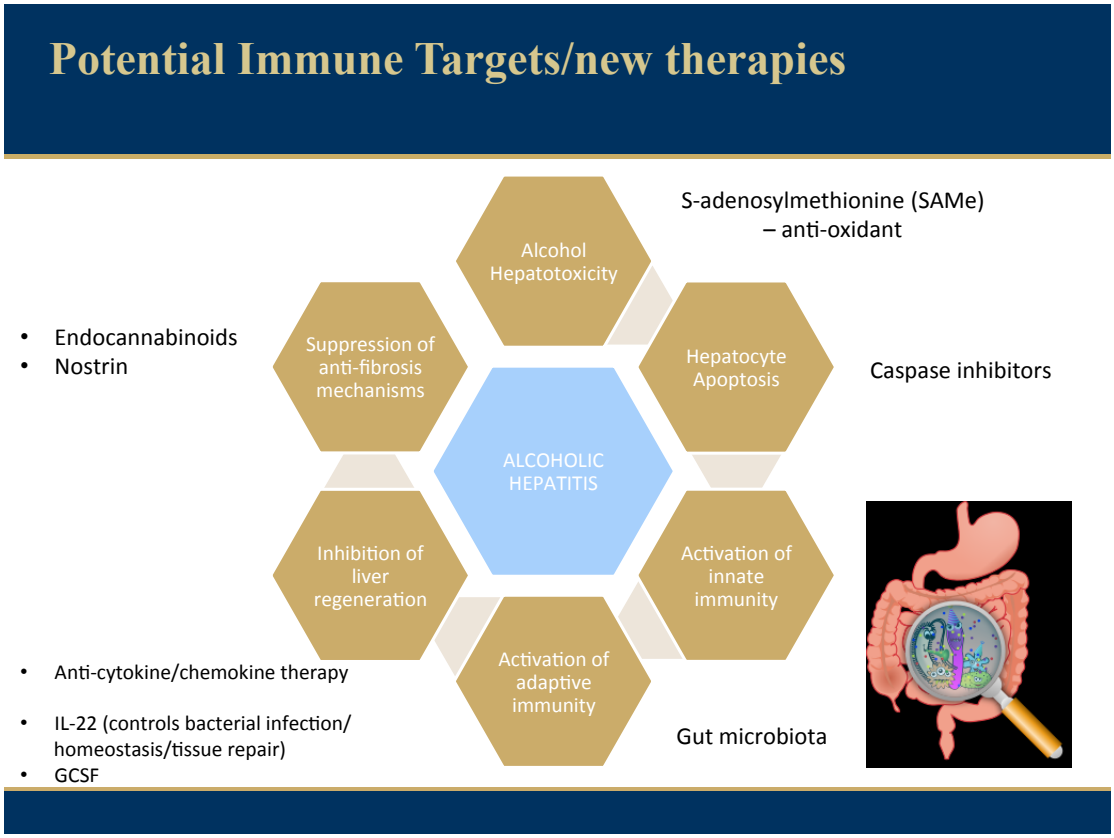


Figure 1.21 Future therapeutic targets and strategies in alcoholic hepatitis

1.8 Synopsis and aims of study

There has been an exponential rise in the incidence of ALD, particularly in the UK. AAH is the most florid form of ALD with a mortality rate of approximately 30% in the first 28 days. Treatment for patients with AAH has not changed in decades. One of the reasons for this is the relative lack of understanding of the pathophysiology and drivers of this condition. No study has longitudinally investigated changes in immune function in this condition and the impact, at this level, of current treatments used.

Sepsis is a significant problem and a major contributor to mortality across the spectrum of liver disease. The rise of multi-drug antimicrobial resistance is further reason to develop non-antibiotic strategies based upon an understanding of the defects leading to increased susceptibility to infection.

Neutrophils are a major immune cell subset involved in the first line of defense against infection; the patient with chronic granulomatous disease highlights the importance of this cell in combating infection. Neutrophil dysfunction, characterised by an inability to phagocytose bacteria, has been described in cirrhosis, there is however a paucity of understanding regarding the mechanisms involved in the neutrophil dysfunction that exists and how this relates to alcohol-induced liver toxicity. In addition, whether other derangements in neutrophil function contribute to the state of immunoparesis in ALD is not well understood.

My preliminary data suggested a 'tolerance' or impaired response to endotoxin stimulation on the one hand and a 'hyper-responsiveness' on the other which may exacerbate the systemic inflammatory response in AAH contributing to poor outcome. The risk of exacerbating the underlying inflammatory state has been

one of the main concerns in the development of immunotherapeutic approaches in AAH. Likewise, anti-inflammatory therapies may render individuals progressively immunosuppressed and this difficult dynamic remains a challenge. Figure 1.22 illustrates my hypothesis; neutrophils are activated by LPS/cytokines/ethanol with subsequent ROS and lactoferrin secretion which may contribute to hepatic inflammation. A cycle of inflammation ensues, the neutrophils become exhausted and fail to appropriately respond to pathogens if encountered. This, of course, is dynamic and the management of the individual is dependent on where they are in the disease course. The impact of current therapies and interplay with the adaptive immune system are also of importance and the topic of study in my work.

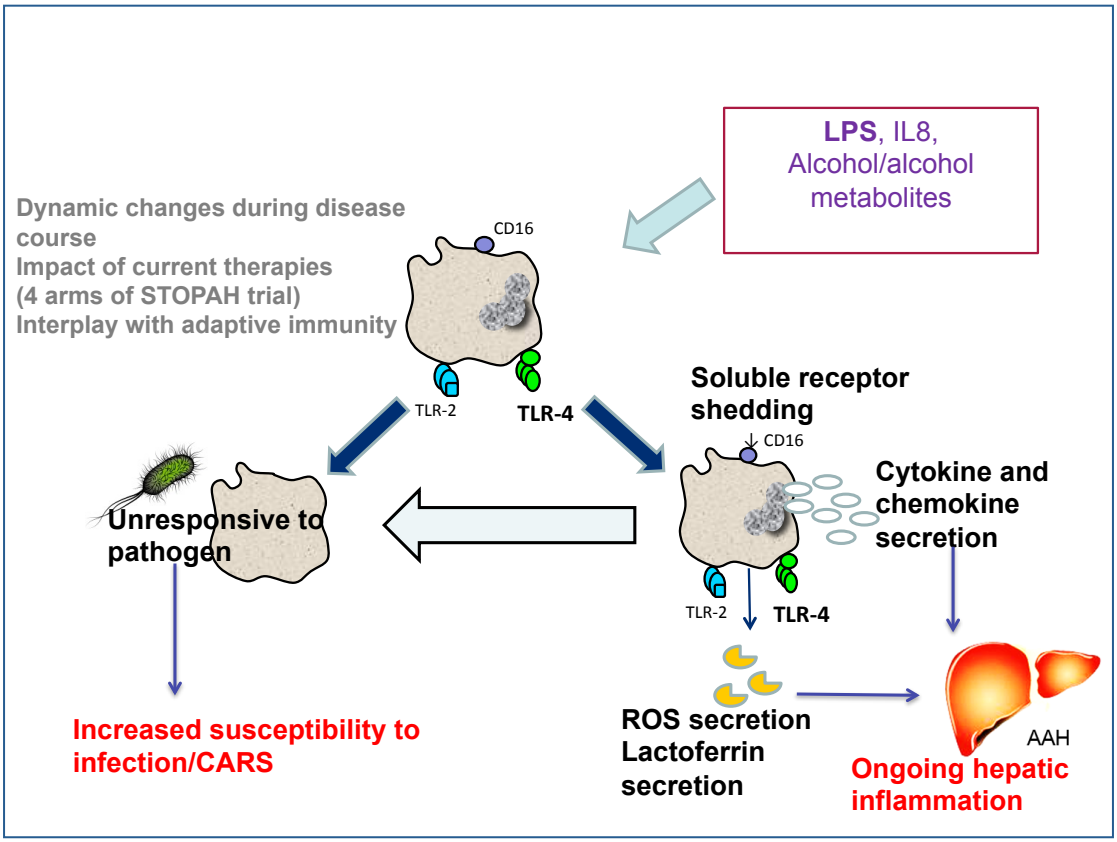


Figure 1.22 Hypothesis of neutrophil dysfunction in alcoholic hepatitis and how this may contribute to hepatic inflammation, SIRS and CARS

My broad intention is to strengthen our knowledge of the immunopathological processes involved in AAH and ARC and potentially improve treatment options and care for this population of patients. The use of the HL-60 cell line, which was originally intended to create part of a co-culture model with a hepatocyte-like cell line, led to further questions regarding neutrophil biology and the work on the neutrophil-interferon-λ relationship both in health and disease states which has, until now, been largely unexplored. As discussed, interferon-λ is of importance at epithelial surfaces and viral liver disease, and given the significance of the gut-liver axis in ALD merited further investigation in this condition.

Aims and objectives:

1. To characterize neutrophil phenotype and responses *ex vivo* to bacterial and LPS challenge in patients with AAH compared with patients with ARC and healthy controls (HC).
2. To evaluate peripherally circulating neutrophils as contributors to hepatic damage and multi-organ failure in AAH compared with patients with ARC and HC.
3. To prospectively examine neutrophil function and the effect of current AAH therapies on innate immune function by sequential analyses of patients enrolled in the STOPAH trial.
4. To examine the interplay between neutrophils, ethanol and the liver by using the HL-60 cell line and an *in vitro* model.
5. To identify novel targets which may propagate immunoparesis in ALD, specifically examining the relationship of neutrophils and IFN-λ in anti-bacterial immune defenses in health and ARC.

2 Materials and Methods

2.1 Materials

2.1.1 General consumables

Rosewell Park Memorial Institute (RPMI) medium [Sigma-Aldrich, United Kingdom (UK)]; Flow cytometer tubes – 5mL [Becton Dickinson (BD), UK]; Falcon tubes / Cellstar tubes - 15 and 50 mL [Starlabs, UK]; Lysing solution [BD]; Polymorphprep™ solution [Axis Shield, Norway]; DNAse-I [Sigma-Aldrich, UK]; Phosphate buffered saline (PBS) [Sigma-Aldrich, UK]; Cytofix/cytoperm solution [BD]; Permwash buffer [BD]; RNAprotect Cell Reagent [Qiagen, UK]

Flow cytometry antibodies: Information given in appendix.

Stimulants: Lipopolysaccharide (LPS) [Sigma-Aldrich, UK]; fixed *E. coli* (strain DH5α); Recombinant interferon-λ1, interferon-λ2, interferon- λ3 [R&D Systems, UK]; Pansorbin [Sigma-Aldrich, UK]; Poly I:C [Invivogen].

Kits: Neutrophil function kit – Phagotest™ and Burst Test™ [Orpegen Pharma, Germany]; Cytometric bead array (CBA) [BD, UK] – Human Soluble Protein Master Buffer Kit [BD, UK]; Human Soluble Protein Flex Sets – IL-6, IL-8, IL-10, TNF-α, GCSF, VCAM-1, ICAM-1, E-selectin, L-selectin, VEGF [BD, UK]; Limulus-ameboctye-lysate assay [LAL, Pierce/ThermoFisher Scientific, UK]; MACSxpress Neutrophil Isolation Kit [Miltenyi Biotec, UK]; Qiagen RNEasy Kit [74104, Qiagen, UK]; QIAamp RNA Blood Mini Kit [52304, Qiagen, UK]; RNeasy MinElute Cleanup Kit [Qiagen, UK]; QuantiTect Reverse Transciption Kit [Qiagen, UK]; Taqman Universal Master Mix II [Applied Biosystems, ThermoFisher Scientific, UK]; Primers and probes for interferon-λ1, interferon-λ2/3, interferon-λ receptor and RPLP0 as endogenous control [Applied Biosystems, ThermoFisher Scientific,

UK]; Lactoferrin ELISA kit (Merck Millipore, Germany); Interferon-λ ELISA kit (R&D Systems, UK].

Instruments: Flow cytometer – BD Fluorescence activated cell sorting (FACS) machine Canto II and LSRII [BD, San Jose, California, USA]; Centrifuge [Rotina, Germany]; Water bath [Grant, UK]; Nanodrop spectrophotometer [Thermo Scientific, UK]; ABI 7500 TaqMan [Applied Biosystems, ThermoFisher Scientific, UK].

Analysis softwares: BD FACS DIVA software V6.0 [BD, San Jose, California, USA]; FCAP array software V1.0.1 [BD, San Jose, California, USA]; SPSS 20.0 [IBM statistics, UK]; GraphPad Prism V6.0 [UK].

2.2 Study design

A prospective cohort study was performed on patients presenting with AAH. A histological diagnosis was not required, this is discussed further in 3.5. Blood samples were collected within 48 hours of hospital admission, prior to administration of any immunosuppressant therapy. Antibiotic use was not a contraindication but patients were not recruited with untreated sepsis. Neutrophil phenotype and function were characterised at baseline and at day 7, 14, 28, 90 and 180; follow-up samples depended on disease-course. Patients with ARC were also sampled, alongside HC.

2.3 Study population

Patients over 18 years of age with severe AAH defined as a bilirubin >80 μmol/L and a Maddrey's Discriminant Factor >32 [36] and a history of excess alcohol (>80g/day male, >60g/day for female), as per the STOPAH study, were recruited. Patients were excluded if there was a history of abstinence from alcohol of >6

weeks, duration of jaundice >3 months, the presence of other causes of liver disease, evidence of current malignancy, pregnancy, the concomitant use of antioxidants or other immunomodulatory therapies within the last 6 months, aspartate aminotransferase >500 IU/L or alanine aminotransferase >300 IU/L, patients with a serum creatinine >500 μmol/L or requiring renal replacement therapy, patients requiring inotropic support, active gastrointestinal haemorrhage and untreated sepsis. Patients with ARC both actively drinking and abstinent from alcohol for greater than 6 months were recruited. ARC patients ranged from Child Pugh A to C, the majority had decompensated disease and were hospitalised, patients with untreated sepsis, malignancy or HIV were not recruited. Healthy subjects were also analysed. The HC alcohol intake was <20g/day and they had not drunk alcohol or exercised excessively 72-hours prior to blood being drawn.

2.4 Consent and data collection

The study was performed in accordance with the declaration of Helsinki and ethical permission was granted from the North East London Research Ethics Committee (reference number 08/H0702/52) and the Steroid or Pentoxifylline for Alcoholic Hepatitis (STOPAH) study (09/MRE09/ 59).

After obtaining fully informed consent or assent; clinical, biochemical and physiological data were collected. Specifically alcohol use, liver biochemistry, synthetic function and the use of antibiotic and immunomodulatory therapies were recorded. Data on the occurrence of bacterial, fungal and viral infection were collected along with the incidence of organ failure and outcome. An application for information on the treatment arms for those patients enrolled in

STOPAH was made after trial completion.

2.5 Sample collection and storage

Venous blood was collected into heparinised pyrogen-free tubes. Neutrophil phenotype and function tests were performed within 2 hours of blood being drawn in all subjects. Plasma was obtained by centrifugation of whole blood at 4500 rpm for 10 minutes at 4°C and stored at -80°C for subsequent analyses including cytokine determination by cytometric bead array (CBA).

2.6 Flow cytometry

The expression [mean fluorescence intensity (MFI) and frequency] of various surface and intracellular receptors present on the white blood cells were analysed using a flow cytometer. Flow cytometry measures optical and fluorescence characteristics of single cells, these pass through a single apparatus in a fluid stream [206]. The size and internal complexity of cells are measured using the light scattered by forward angle and right angle respectively. Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on the cell surface or inside the cell. Samples were acquired on a BD FACSCanto II ™.

2.7 Isolation of white blood cells after lysis of red cells

One hundred μL of whole blood was placed in 5 mL tubes and 1 mL of lysis solution [<15% formaldehyde and <50% di-ethylene glycol] was added. The lysate was mixed gently and left at room temperature (RT) for 15 minutes. The reaction was stopped by adding 2 mL of phosphate-buffered saline (PBS). The solution was centrifuged at 600g for 5 minutes at 18°C and the supernatant

discarded leaving the cell pellet at the bottom of the tube. The cells were then re-suspended in 100 μL of PBS before staining for flow cytometry.

2.8 Isolation of white blood cells using Polymorphprep™ solution

Polymorphprep™ solution separates white blood cells and other blood components based on their density. To isolate the white blood cells using Polymorphprep™ solution, 20 mL of heparinised blood was collected and processed under aseptic conditions in a bio-safety cabinet. Whole blood was layered over 15 mL of Polymorphprep™ solution in a 50 mL falcon tube. The tubes were balanced and centrifuged at 800g for 35 minutes at 18°C, with least acceleration and zero brakes. The separated blood contains peripheral blood mononuclear cells (PBMC) on top, granulocytes in the middle and red blood cells at the bottom (Figure 2.1). PBMC and granulocytes were isolated in two different sterile 50 mL falcon tubes. Granulocytes were washed twice with PBS and centrifuged at 800g for 10 minutes at 18°C with brakes.

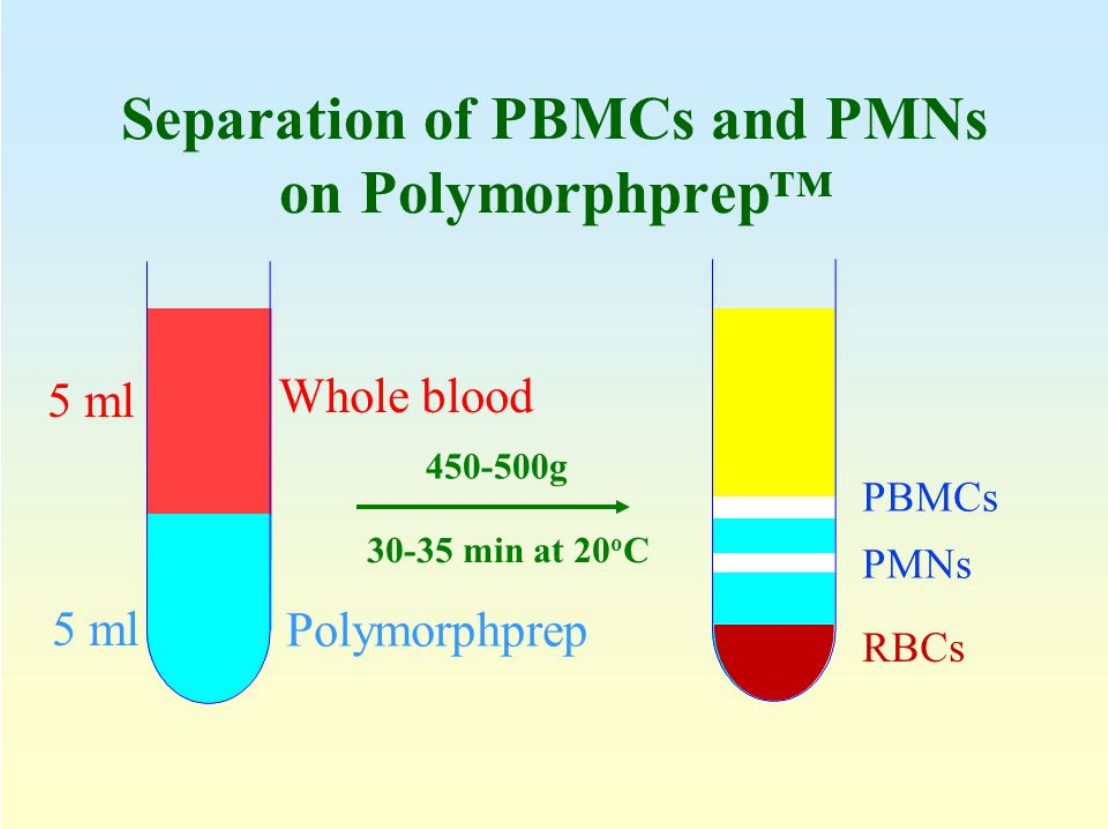


Figure 2.1 Separation of PBMCs and PMNs on Polymorphprep™

2.9 Isolation of neutrophils using the MACSxpress Neutrophil Isolation Kit

The MACSxpress neutrophil isolation kit isolates untouched neutrophils directly from whole blood without density gradient centrifugation. Red blood cells are aggregated and sedimented, while non-target cells are removed by immunomagnetic depletion with MACSxpress Beads.

The MACSxpress Neutrophil Isolation Cocktails is delivered as a lyophilized pellet. The pellet was reconstituted by adding 2 mL of Buffer A to one vial of lyophilized MACSxpress Cell Isolation Cocktail and mixed gently immediately before use. The final cocktail was prepared by mixing the appropriate volumes of reconstituted pellet and Buffer B. For 8 mL of blood, the final cocktail was prepared by adding 2 mL of reconstituted pellet to 2 mL of Buffer B and mixed gently. The 4 mL cocktail was added to the 8 mL of blood in a 15 mL tube. The

tube was closed tightly and inverted gently three times. The sample was incubated for 5 minutes at room temperature on a tube rotator on run speed of 12 rpm. The tube was removed from the tube rotator and the cap carefully opened. The open tube was placed in the magnetic field of the MACSxpress Separator for 15 minutes (Figure 2.2).

Whilst the tube was inside the MACSxpress Separator, the supernatant was carefully collected into a new 15 mL tube. The supernatant contained the target cell fraction. An aliquot of the supernatant was taken for cell counting.

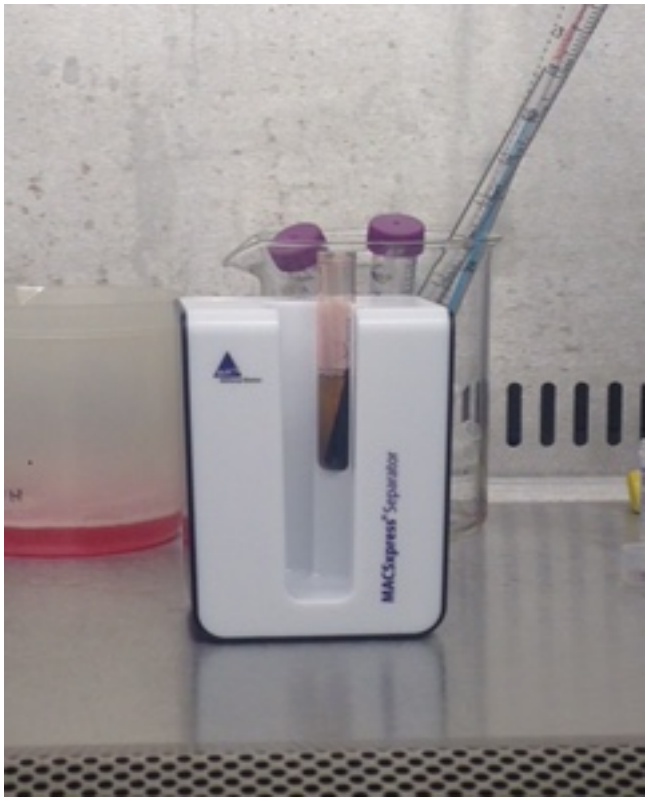


Figure 2.2 The MACSxpress Separator.

The magnetically labeled cells will adhere to the wall of the tube while the aggregated erythrocytes sediment to the bottom.

2.10 Cell count

The cells were suspended with PBS and diluted (1/10) with Trypan Blue solution. Cells were counted using a Neubauer haemocytometer and up to 1 million ($1x10^6$) viable cells were re-suspended with 100 µL of PBS in a single tube for flow cytometry staining.

The total number (no.) of cells in the PBS (isolated from a single sample) was calculated as follows:

Total no. of cells = Cells counted x dilution factor x depth factor x 10^3 x volume of PBS / Area counted

= Cells counted x 10 x 10 x 10^3 x volume of PBS / 4

Cells counted – number of cells counted in the haemocytometer.

Dilution factor – the dilution of cells with trypan blue (1 in 10 dilution – 5 µL cells with 45 µL of trypan blue).

Depth factor – the distance between the cytometer and cover slip (0.1 mm - 10)

10^3 – for the conversion of per mL.

Volume of PBS – 5 mL of PBS in which the cells were suspended.

Area counted – four, the 16 small squares in the areas 1, 2, 3 and 4 of the haemocytometer were counted as shown below in figure 2.3.

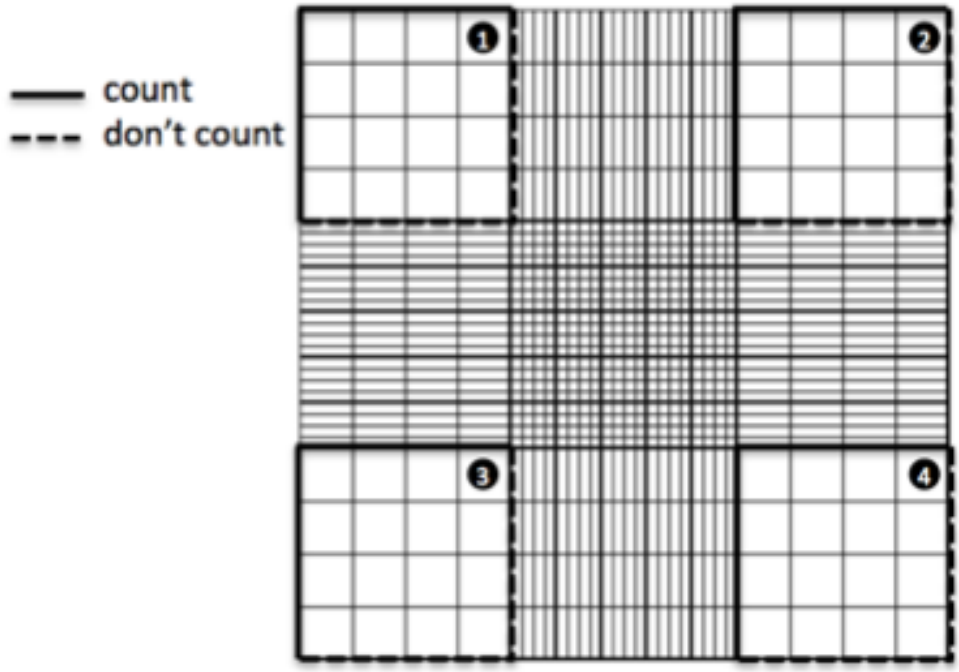


Figure 2.3 Image of the areas used for counting white blood cells in a haemocytometer

2.11 Cell surface and intracellular receptor analyses

2.11.1 Staining of cells for flow cytometry

For flow cytometry, up to 100, 000 white blood cells were re-suspended in 100 µL of staining buffer and stained with fluorochrome conjugated antibodies in one tube. To stain for receptors present on cell surface, monoclonal antibodies conjugated with fluorochromes were added to the white blood cells, mixed and incubated at RT in darkness for 30 minutes. Following this the cells were washed with 1 mL of sterile PBS, centrifuged at 600g for 5 minutes at 18°C and the supernatant was discarded.

To prepare the cells for intracellular staining, 100 µL of cytofix/cytoperm solution was added to the cell pellet after surface stain, mixed gently and left at RT for 20 minutes. The cells were washed with 1 mL of BD permeabilisation wash buffer, centrifuged at 600g for 5 minutes at 18°C and the supernatant was discarded. The cells were then stained with intracellular or intranuclear antibodies and incubated at RT in darkness for 30 minutes. The cells were washed again with 1 mL of permeabilisation buffer, centrifuged at 600g for 5 minutes at 18°C and the supernatant was discarded. The cells were then re-suspended in 300 µL of PBS and analysed using a flow cytometer.

All antibodies were titrated by staining them with blood samples collected from HC to determine the right concentration of antibody required to be added to the cells. The stock antibodies were diluted with PBS and those dilutions which expressed maximum mean fluorescence intensity (MFI) were chosen for the experiment.

2.11.2 Identification of white blood cells and the different subsets

The BD FACSCanto II ™ instrument was used and operated using the BD FACS Diva Software 6.1.2. A new experiment was created and compensation settings applied. In a new global worksheet, a dot plot was created to display the cells. Cells were identified in the forward scatter channel area (FSC-A) and side scatter channel area (SSC-A) of the dot plot based on their size and complexity. As cells increase in size and complexity they move away from zero in FSC-A and SSC-A respectively. The FSC-A and SSC-A are measured on a linear scale and the fluorochrome expression was measured on a logarithmic scale.

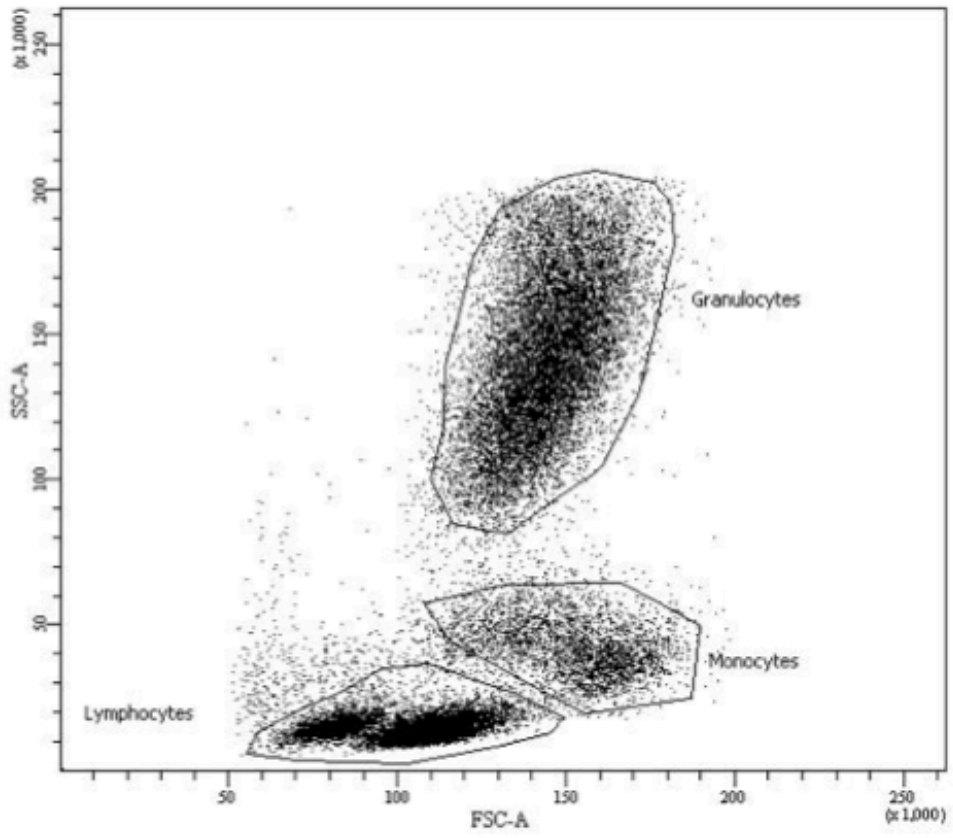


Figure 2.4 Flow cytometry image of human leukocytes showing the position of granulocytes, monocytes and lymphocytes on a FSC-A/SSC-A dot plot.

Figure 2.4 demonstrates the leukocyte populations. The lymphocytes are small cells without granules hence their position on the dot plot. The population further along the X-axis are considered as monocytes since they are the large cells amongst the white blood cells without granules. The large population above are considered as granulocytes, since they are present in the majority amongst the white blood cells and contain granules. The different populations, namely the granulocytes in my work, were further characterised by staining for the key receptors expressed on those cells using fluorochrome conjugated antibodies.

2.11.3 Identification and characterisation of neutrophils

In humans, CD16, CD11b and CD62L have been shown to be useful markers for the identification of neutrophils. Depending on the experiment set-up, these markers were used as an individual marker or in combination to identify the neutrophils. (CD16, an Fc-gamma receptor, CD11b, a sub-unit of the integrin receptor MAC-1 and CD62L or L-selectin, another adhesion molecule are discussed in 1.5.) Baseline neutrophil phenotype was characterised by determining the expression of CD16, CD11b, TLR2, TLR4 and TLR9 in granulocytes isolated from whole blood after erythrocyte lysis.

2.11.4 Identification and characterization of T cells

T cells were identified using CD3 an important receptor present on the surface of T cells that helps in its activation. Following that, CD4 and CD8 markers were used to characterize the different subsets of T cells.

2.12 Neutrophil phagocytosis

2.12.1 Principle and assay procedure

Phagocytosis was quantified using the Phagotest™ which allows the quantitative determination of leukocyte phagocytosis using fluorescein isothiocyanate (FITC)-labelled opsonised *E. coli* bacteria on the flow cytometer. One hundred µL of whole blood was incubated in a water bath at 37°C in darkness for 20 minutes with 20 µL of FITC labelled opsonised *E. coli* along with a control (PBS). Ice cold trypan blue was added to all the tubes and left at RT for 20 minutes to quench the fluorescence of bacteria attached to the cell surface. Cells were washed with 2 mL PBS by gently mixing with a pipette, centrifuged at 600g for 5 minutes and the supernatant was discarded. Red cell lysis was performed by adding 2 mL of lysis solution with an incubation period of 15 minutes at room temperature. Cells were then washed with 2 mL PBS and centrifuged as above and the supernatant discarded. Four µL of fluorochrome conjugated antibody CD16 (PE) was added to the tube containing FITC-labelled *E. coli* and the control tube and incubated at RT in darkness for 30 minutes. Cells were washed with 2 mL of PBS, centrifuged at 600g for 5 minutes and the supernatant was discarded. Three hundred µL of PBS was added to all the tubes and acquired in a flow cytometer. A negative control tube (100 µL of whole blood) was treated in similar conditions without the addition of FITC-labelled *E. coli* and CD16-PE.

2.12.2 Flow cytometer set-up for the neutrophil phagocytosis assay (NPA)

A new experiment was created and the specimen tubes were labelled as negative, tube – 1 and tube – 2. In the inspector, settings were adjusted to show FSC-A, SSC-A, FITC and PE in the panel. In the global worksheet, a dot plot with

FSC-A (X-axis) and SSC-A (Y-axis) was created. Cells in the negative tube were acquired and the FSC/SSC voltages were adjusted between 250 - 400 V to identify the white blood cells in the dot plot. The large population in the dot plot was identified as granulocytes [Figure 2.5]. Another dot plot with CD16-PE (X-axis) and SSC-A (Y-axis) were created showing the granulocytes. While cells from tube – 1 (without FITC *E. coli*) were acquired, granulocytes expressing CD16+ were gated and identified as CD16+ neutrophils. A plot with FITC *E. coli* showing CD16+ neutrophils was created. An interval gate was fixed using the FITC-negative/control population (tube 1) [Figure 2.6]. Cells from tube – 2 were acquired with the voltages adjusted based on tube – 1 to identify the neutrophils undergoing phagocytosis (%) [Figure 2.7].

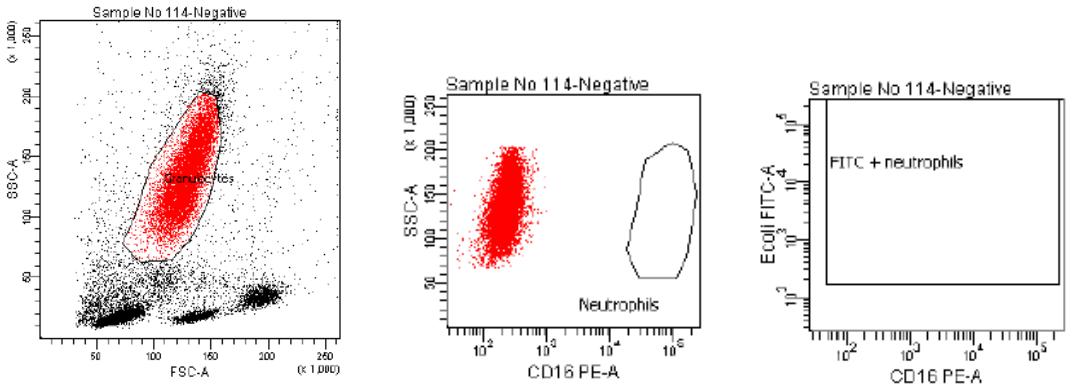


Figure 2.5 Image of dot plots from negative tube in NPA.

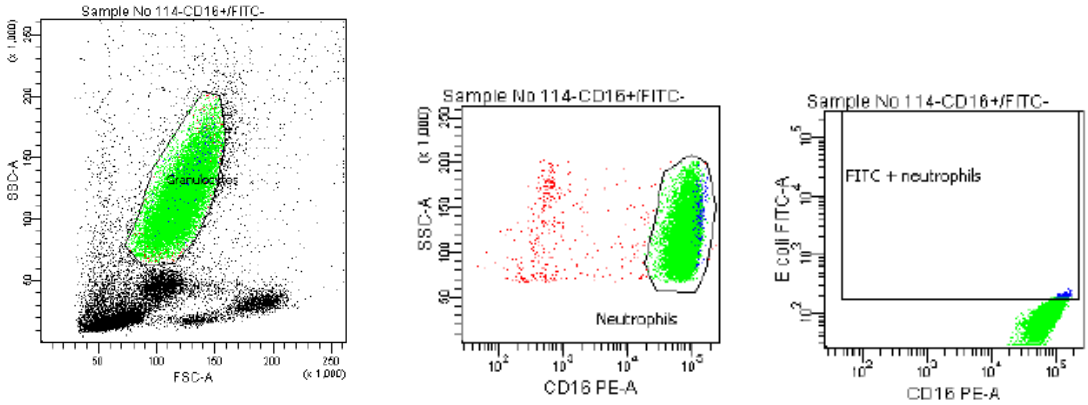


Figure 2.6 Image of dot plots from tube 1 in NPA.

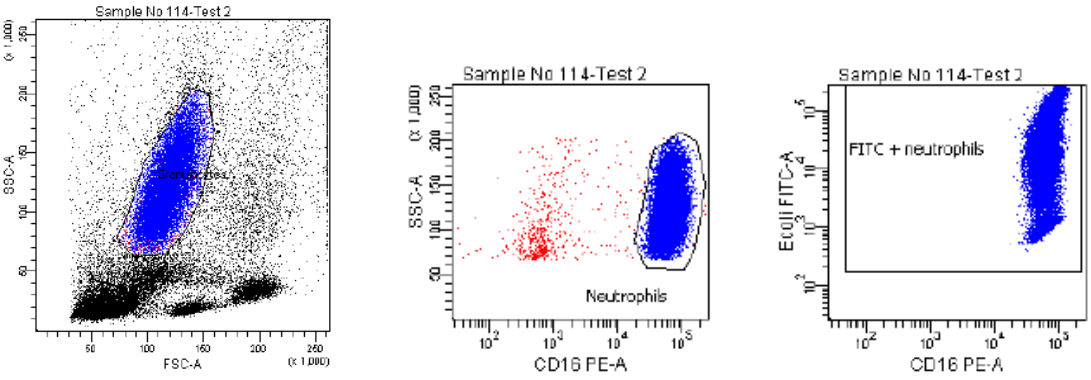


Figure 2.7 Image of dot plot from tube 2 in NPA.

2.13 Neutrophil oxidative burst

2.13.1 Principle and assay procedure

Neutrophil oxidative burst (OB) was determined with a Burst kit using different stimulants and dihydrorhodamine as a fluorogenic substrate. The percentage of phagocytic cells which produce reactive oxygen species (ROS) (the conversion of dihydrorhodamine to rhodamine and the amount of rhodamine per cell) can be quantified using a flow cytometer.

Neutrophil low burst (LB) was assessed by stimulating the whole blood with 20 µL of chemotactic synthetic peptide, formyl-Met-Leu-Phe (fMLP) (0.2µM) and high burst (HB) was assessed by stimulating whole blood using 20 µL of protein kinase C ligand phorbol 12-myristate 13-acetate (PMA) (0.2µM). Neutrophil phagoburst (PB) was assessed by stimulating the whole blood with 20 µL of *E. coli* ($2x10^7$). Resting oxidative burst (OB) was assessed by adding 20 µL of PBS to the whole blood. To measure the ROS production, 20 µL of dihydrorhodamine was added to all the tubes and incubated in a water bath at 37°C in darkness for 20 minutes. Dihydrorhodamine-123 is oxidised to rhodamine-123 in the presence of ROS and gives green fluorescence. Whole blood without dihydrorhodamine was added to a tube separately as a control. Cells were washed with 1 mL of PBS by gently mixing with a pipette, centrifuged at 600g for 5 minutes and the supernatant was discarded. Red blood cells were lysed using a lysis solution as described above. Four µL of fluorochrome conjugated antibody CD16 (PE) was added to all the tubes and incubated at RT in darkness for 30 minutes. Cells were washed with 2 mL of PBS, centrifuged at 600g for 5 minutes and the supernatant was discarded. Three hundred µL of PBS was added to the

tubes and acquired in a flow cytometer. A negative control (100 µL of whole blood) was treated in similar conditions without the addition of dihydrorhodamine and CD16-PE.

2.13.2 Flow cytometer set-up for the neutrophil oxidative burst experiment

The experiment was set-up using the methods described for the NPA - the rhodamine positive population was set using the negative control.

2.14 Lipopolysaccharide stimulation

Whole blood was incubated at 37°C with Roswell Park Memorial Institute (RPMI)-1640 media and stimulated with either LPS (*E. coli* 0111:B4) (200ng/mL) or with PBS in controls for 2 hours (unstimulated). The stimulated cells were then stained and analysed by flow cytometry as previously described. The supernatant of the stimulated cells were carefully collected and stored at -80°C for subsequent cytokine analysis. The conditions used for the *ex-vivo* studies, including the concentration of LPS had previously been optimised using sequential assays of different concentrations of stimuli with healthy blood.

2.15 Endotoxin measurement

The Toxisensor Chromogenic Limulus Amoebic Lysate (LAL) Endotoxin Assay (Genscript, UK) was used to measure the endotoxin levels in the plasma samples stored at -80°C. This kit can measure from 0.005 to 1 endotoxin unit / mL (EU/mL) and detects endotoxin based on a chromogen using a modified LAL and a synthetic substrate. This assay was performed using the protocol booklet provided with the kit.

2.16 Lactoferrin measurement

Sandwich ELISA was performed as per protocol, using a Lactoferrin ELISA kit (Merck Millipore, Germany).

2.17 Analyses of cytokines and endothelial activation markers

Pro- and anti-inflammatory cytokines [IL-8, IL-6, IL-10, TNF-α, GCSF] levels and endothelial activation markers [VCAM-1, ICAM-1, E-selectin, L-selectin, VEGF] were determined in previously stored plasma and supernatant samples using cytometric bead array (CBA). Cytokine levels were quantified using the capture and detection beads from different Human Soluble Protein Flex Sets and a Master Buffer Kit.

2.17.1 Preparation of standards

A lyophilised standard from the flex set of each cytokine tested were pooled into one 15 mL polypropylene tube and reconstituted with assay diluent. The reconstituted standards were left to equilibrate for 15 minutes at RT followed by gentle mixing using a pipette. A serial dilution was performed by transferring 500 µL of top standard with equal volume of assay diluent to 1:2 dilution and then further up to 1:256. Assay diluent was used as the 0-pg/mL.

2.17.2 Preparation of capture and detection beads

Based on the instruction manual provided, equal volumes (50 µL) of capture bead and detection bead were used for testing the plasma or supernatants and the beads were 50x concentration. This experiment was optimised in our laboratory; it was found that the capture and detection beads yield a good result when diluted 100x. All the beads were vortexed for 15 seconds immediately

before use.

2.17.2.1 Capture beads

As per protocol, capture bead preparation varied depending on whether supernatant samples or plasma samples were being analysed. The total bead volume was calculated based on the number of samples (including standards) and cytokines measured for each experiment.

For plasma samples:

The appropriate amount of capture beads (as shown below) from the stock vials of each Human Soluble Protein Flex Set were pooled into a 15-mL polypropylene tube and 0.5 mL of wash buffer was added to them. The tube was then centrifuged at 200 g for 5 minutes and the supernatant was discarded by carefully aspirating it without disturbing the bead pellet. The bead pellet was then re-suspended in capture bead diluent to a final concentration of 50 µL / test.

An example calculation:

No. of tests – 50; Volume of capture beads from each flex set – 25 µL; No. of flex sets (cytokine beads) – 4; final concentration – 50 µL

Therefore Total bead volume = 50

No. of tests x 50 µL of final concentration = 2500 µL

For supernatants:

The appropriate amount of capture beads (as shown below) from the stock vials

of each Human Soluble Protein Flex Set were pooled into a 15-mL polypropylene tube and mixed with capture bead diluent. The final concentration of capture bead volume was 50 µL / test. The volume of the capture bead diluent was calculated by subtracting the volume for each bead tested from the total bead volume required for the assay.

An example calculation:

No. of tests – 50; volume of capture beads from each flex set – 25 µL; final concentration – 50 µL; No of flex sets (cytokine beads) – 4

Total bead volume = No. of tests – 50 x Final concentration – 50 µL = 2500 µL

So when 4 cytokines were tested, diluent volume = 2500 µL – (25 µL x 4) = 2400 µL.

2.17.2.2 Detection beads

The procedure for detection bead preparation was the same for supernatant samples and plasma. Therefore the appropriate amount of detection beads (as shown below) from the stock vials of each Human Soluble Protein Flex Set were pooled into a 15-mL polypropylene tube and mixed with detection bead diluent. The final concentration of detection bead volume was 50 µL/test. The volume of the detection bead diluent was calculated by subtracting the volume for each bead tested from the total bead volume required for the assay.

An example calculation:

No. of tests – 50; Volume of detection beads from each flex set – 25 µL; No of flex sets (cytokine beads) – 4; Final concentration – 50 µL

Total bead volume = No. of tests – 50 x Final concentration – 50 µL = 2500 µL

When 4 cytokines were tested, diluent volume = 2500 µL – (25 µL x 4) = 2400 µL

2.17.3 Assay procedure

The assay was performed on tubes and 50 µL of flex standards starting from 0 to 2000 pg/mL (top standard) and plasma and supernatant samples were placed in FACS tubes. The mixed capture beads were vortexed for at least 5 seconds and 50 µL was added to the standards and samples. The tubes were mixed gently and incubated at RT in darkness. After an hour, the mixed PE detection reagent was vortexed for at least 5 seconds and 50 µL was added to all the tubes. The tubes were mixed gently and left to incubate at RT in darkness. After two hours of incubation, 1 mL of wash buffer was added to all the tubes and centrifuged at 200g for 5 minutes. The supernatant was discarded carefully and the beads were re-suspended in 300 µL of wash buffer for flow cytometry analysis.

2.17.4 Sample acquisition and analyses

All samples were acquired in the BD FACS CantoII flow cytometer using the BD FACS DIVA software. Compensation was performed using the instrument setup beads provided along with the Master Buffer Kit, to avoid spectral overlap. Standards were acquired starting from negative or 0-pg/mL to top standard or 2000 pg/mL followed by the plasma or supernatant samples. Files were saved as FCS 3.0 and the results were analysed using the FCAP array software.

2.18 HL-60 cell culture

The HL-60 cells were sourced from the American Type Culture Collection (ATCC). HL-60 cells are distributed as frozen aliquots of low passage cells in RPMI-1640 with 10% glycerol and are stored in liquid nitrogen. Cells were thawed and re-suspended in culture medium. To make the culture medium 11.5 mL of 1M HEPES buffer, 3 mL of 1M sodium hydroxide, 5 mL of Penicillin

Streptomycin and 500 µL of Glutamine were added to 1000 mL of RPMI. The RPMI was then supplemented with 20% fetal bovine serum (FBS). Cells were maintained initially in 20% FBS, then transferred to 10% FBS and maintained between 0.1-1 million cells/mL.

2.19 HL-60 differentiation and characterisation

Different concentrations of DMSO were used to induce neutrophil differentiation. After characterisation and examination of function data, the decision was made to use 1.5% DMSO and examine cells at day 6 of exposure.

The expression of CD14, CD16, CD11b, TLR2, TLR4, TLR9, CD181 and CD182 on the differentiated and non-differentiated cells were examined by FACS as outlined earlier.

The Phagotest and Phagoburst experiments were adapted for use on the differentiated cells.

The function of differentiated HL-60 cells was also assessed following culture with ethanol in concentrations of 10mM, 50mM, 100mM, 250mM and 500mM. In these experiments, hepatocyte (VL17A) metabolized ethanol was also used (courtesy of Dr Elena Palma Institute of Hepatology).

2.20 Evaluation of interferon lambda

2.20.1 High sensitivity IFN-λ1 ELISA

HC and patient plasma were examined for IFN-λ1 using a sandwich ELISA, performed as per protocol (High sensitivity IFN-λ1 ELISA kit, R&D Systems, UK).

2.20.2 Staining for lambda receptor in healthy control leucocyte subsets

Two hundred μL of blood from HCs was drawn and 100 μL incubated with CD14, CD16, CD11b, CD3, CD56, CD19 plus IL28 receptor isotype or IL28 receptor. After 15 minutes, samples were lysed and washed as described above, then examined using FACS.

2.20.3 Interferon lambda and HL-60 cells

Differentiated and non-differentiated HL-60 cells were stained for IL28 receptor and examined using FACS. Differentiated HL-60 cells were incubated with recombinant IL29 (100 ng/mL) and phagocytosis tested as described earlier.

2.20.4 Healthy control stimulant optimisation

1.5 mL of HC blood was cultured in duplicates with either 15 μL of Pansorbin (1:1000), 150 μL of fixed E. coli (10 bacteria/cell), 75 μL of Poly I:C or PBS as control. After 2 hours, one set of the above samples underwent neutrophil extraction using the MACSxpress Neutrophil Isolation Kit (2.9) providing a neutrophil fraction for RNA extraction. RNA was extracted directly from the other 4 samples using the Qiagen Blood Mini RNA kit (the leukocyte fraction).

As neutrophils have a low RNA content [207], volumes of blood (and accordingly stimulant) were increased to 8 mL to increase the yield of neutrophil RNA. The leukocyte cultures were kept at 1.5 mL.

2.20.5 RNA extraction

To extract RNA from isolated neutrophils and the HL-60 cells the Qiagen RNEasy Kit (74104, Qiagen, UK) was used, and to extract RNA from whole blood cultures the QIAamp RNA Blood Mini Kit (52304, Qiagen, UK) was used. Extracted RNA

samples were concentrated with the RNeasy MinElute Cleanup Kit (74204, Qiagen, UK) according to manufacturer's instructions.

2.20.6 PCR

RNA was transcribed into cDNA using the QuantiTect Reverse Transcription Kit (205311, Qiagen, UK). Quantitative PCR was carried out using specific primers and probes for interferon-λ1, interferon-λ2/3, interferon-λ receptor and RPLP0 as endogenous control (Applied Biosystems, ThermoFisher Scientific, UK). Fifty nanograms of cDNA were used per reaction. Reactions were performed in duplicate on an ABI 7500 TaqMan. Relative mRNA amounts were quantified using the ΔΔCt method with normalization to RPLP0 gene expression.

2.20.7 Investigation of interferon lambda and neutrophil function in ALD

Patients with ARC were recruited. Plasma was obtained by centrifugation at 3000g for 10 minutes at 4°C and stored at -80°C for subsequent cytokine analyses by Cytokine Bead Array (CBA) or enzyme-linked immunosorbent assay (ELISA).

Whole blood was incubated with fixed *E. coli* (strain DH5α, grown in LB broth and fixed with 1% paraformaldehyde in sterile phosphate buffered saline, PBS) at a concentration of 10 bacteria/cell or PBS (unstimulated) at 37°C for 2 hours. Two parallel cultures were set up and after 2 hours neutrophils were isolated immediately as described previously for RNA extraction, supernatants collected and the whole blood fractions (both stimulated and unstimulated) were stored in RNAprotect Cell Reagent (Qiagen, UK) at -80°C for subsequent RNA extraction. RNA extraction and PCR were performed as described above.

Patient whole blood was incubated individually with recombinant interferon-λ1, interferon-λ2 or interferon-λ3 at a concentration of 100ng/mL (R&D Systems, UK) for 2 hours at 37°C. PBS was used as negative control. Supernatants were collected by centrifugation and stored at -80°C for subsequent cytokine quantification. Fluorochrome-conjugated antibodies (anti-human CD182, PSGL-1, CD11b, TLR4, TLR2, CD16, CD14, CD62L; BD Biosciences, UK; eBioscience, UK) were used for staining and analysed by flow cytometry. Phagotest and Phagoburst kits were used to measure neutrophil phagocytosis and production of reactive oxygen species pre- and post-culture with interferon-λ.

High sensitivity IL29 ELISA was performed on plasma and supernatant samples.

2.21 Statistical analyses

Patient demographics are expressed as median (inter-quartile range), frequency (percentage) as appropriate. Data were analysed using independent or paired *t* test, analysis of variance or non-parametric tests where appropriate. Pearson or Spearman rank correlations were used as appropriate. Statistics were calculated using MS Excel 2010, SPSS 21 or GraphPad Prism 6. *p* value less than 0.05 was considered as statistically significant.

3 Neutrophil phenotype, function and responses to bacterial challenge in alcohol-related liver disease

3.1 Background

As outlined in chapter 1, neutrophil dysfunction has been recognized in AAH and ARC for some time. The risk of exacerbating the underlying inflammatory state has been one of the main concerns in the development of immunotherapeutic approaches in AAH. Likewise, anti-inflammatory therapies may render individuals progressively immunosuppressed and this difficult dynamic remains a challenge. Neutrophil function was therefore examined alongside plasma cytokine determination with the aim of providing a comprehensive profile of the changes seen in AAH patients at admission. The comparative groups used were HCs, and patients with ARC. For some analyses the latter group were divided into those who were actively drinking and those who were abstinent from alcohol to provide further information on the impact of active alcohol consumption on the parameters examined. The neutrophil response to LPS was examined to mimic an infective insult or 'second hit' frequently seen in these groups of patients. In addition, the inhibitory checkpoints PD1 and TIM-3, described in 1.3.5, were examined on innate and adaptive immune compartments, the relationship with LPS and effect of blockade of these receptors on neutrophil function was also specifically evaluated.

3.2 Aim of the investigation

To characterize neutrophil phenotype and responses *ex vivo* to bacterial and LPS challenge in patients with AAH compared with patients with ARC and HCs. Neutrophil phagocytosis, stimulated oxidative burst, plasma endotoxin levels, and neutrophil receptor response and cytokine production following LPS exposure were examined.

To evaluate peripherally circulating neutrophils as contributors to hepatic damage and multi-organ failure in AAH compared with patients with ARC and HCs. Neutrophil resting burst, cell surface receptor changes, plasma lactoferrin, cytokine and endothelial activation markers were examined.

To evaluate whether any defects in neutrophil function had reversibility following the examination and blockade of the immune inhibitory checkpoints PD1 and TIM-3.

3.3 Methods

3.3.1 Patients and study design

A prospective cohort study was performed on patients presenting with severe AAH as described in 2.3. ARC patients and HCs were also recruited as described.

3.3.2 Neutrophil phenotype

Baseline neutrophil phenotype was characterised by determining the expression of CD16, CD11b, TLR2, TLR4 and TLR9 in granulocytes isolated from whole blood after erythrocyte lysis as described in 2.11.

3.3.3 Neutrophil function

Baseline neutrophil phagocytosis and oxidative burst were characterised as described in 2.12 and 2.13.

3.3.4 Endotoxin analyses

The Toxisensor Chromogenic Limulus Amoebic Lysate (LAL) Endotoxin Assay (Genscript, UK) was used to measure the endotoxin levels in the plasma samples stored at -80°C.

3.3.5 Lipopolysaccharide stimulation

As described in 2.14, whole blood was incubated at 37°C with Roswell Park Memorial Institute (RPMI)-1640 media and stimulated with either LPS (*E. coli* 0111:B4) (200ng/mL) or with PBS in controls for 2 hours (unstimulated). The stimulated cells were then stained and analysed by flow cytometry. The supernatants of the stimulated cells were carefully collected and stored at -80°C for subsequent cytokine analysis.

3.3.6 Lactoferrin quantification

Sandwich ELISA was performed as per protocol, using a Lactoferrin ELISA kit (Merck Millipore, Germany).

3.3.7 Analyses of cytokines and endothelial markers

Pro- and anti-inflammatory cytokines [IL-8, IL-6, IL-10, TNF-α, GCSF] levels and endothelial activation markers [VCAM-1, ICAM-1, E-selectin, L-selectin, VEGF] were determined in previously stored plasma and supernatant samples using CBA as described in 2.17.

3.3.8 The effect of Programmed cell death 1 (PD1) and T-cell immunoglobulin and mucin domain-containing protein 3 (TIM3) blockade on neutrophil function

Expression of PD1/PDL1 and TIM3/galectin-9 were assessed by FACS on immune cell subsets in whole blood and PBMCs. For blocking experiments, neutrophils were pre- incubated with 10 mg/mL anti–TIM3 and anti–PD1 for 4 hours at 37^0C/5% CO_2 and Phagotest and Phagoburst (Orpegen Pharma, Germany) were adapted for use in 96-well plates and analyzed by FACS according to previously described protocols. CBA was performed on PBMC

culture supernatants for production of IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, TNF-α and interferon gamma as previously described.

3.4 Results

3.4.1 Study population

Thirty-one patients with severe AAH were recruited. Thirty-one patients with ARC were recruited, sixteen were actively drinking (AA), fifteen patients were abstinent (ABA), defined as no consumption of alcohol for greater than 6 months. Ten HC were analysed (Figure 3.1.) The HC were recruited from within the department, these subjects were not age-matched to the disease groups and this is discussed further in 3.5.

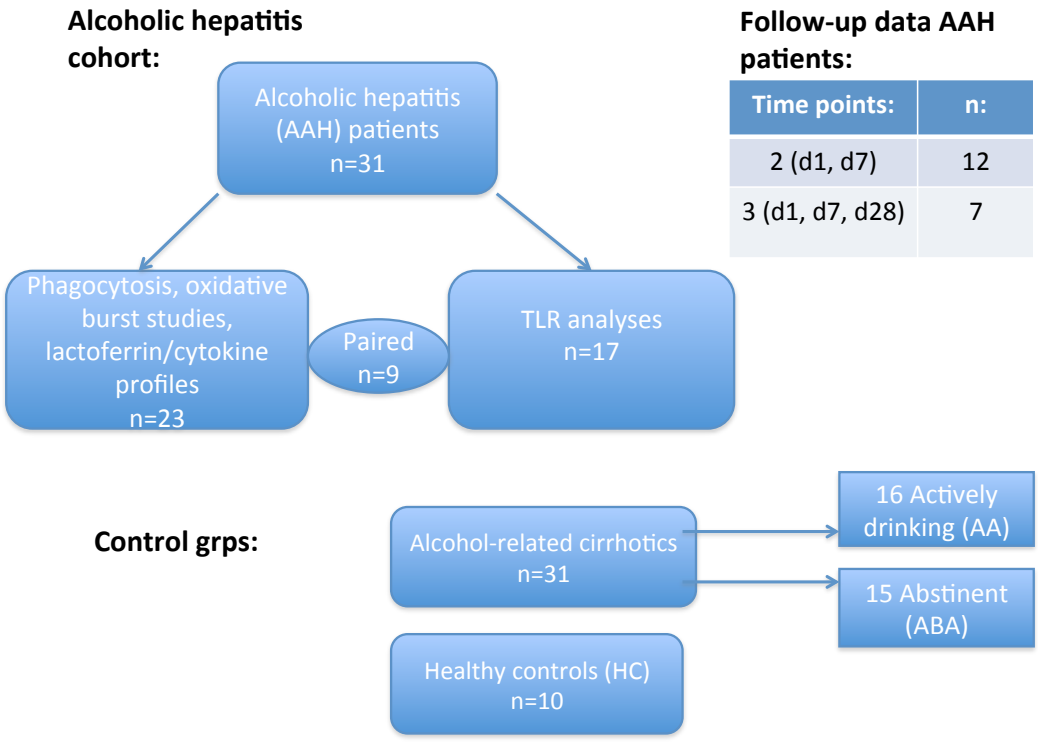


Figure 3.1 Patient groups, analyses performed and time points of sequential analyses in the alcoholic hepatitis cohort.

9 patients had 'paired' analyses where both neutrophil function and TLRs were both assessed.

Baseline demographics, biochemical and physiological parameters are detailed in Table 3.1. As expected, the patients with AAH had significantly higher MELD scores and higher mortality rates than the patients with ARC. Eighteen out of 31 AAH patients were on antibiotic therapy at the time of sampling. Seven out of the 31 ARC patients were graded as Child-Pugh A, the remainder were Child-Pugh B or C and recruited whilst inpatients. Twelve out of the 31 ARC patients were on antibiotics at the time of sampling, in the context of suspected infection patients were not recruited until they had received 48 hours of antibiotics. Table 3.2 gives some further clinical information on the ARC cohort. Table 3.3 details the positive culture results obtained in individual patients. Eleven out of 26 patients with AAH received prednisolone, (treatment data was unknown in five patients discussed in 4.5) and this is discussed in further detail in chapter 4.

Table 3-1 Demographic and clinical data for patients studied

Parameters Median (IQR)	**HC (n=10)**	**AAH (n=31)**	**AA (n=16)**	**ABA (n=15)**
Age	36 (29-44)	49 (34-60)	51 (44-54)	56 (48-60)
Female (%)	4 (40%)	17 (55%)	5 (31%)	6 (40%)
Bilirubin (3-20 µmol/L)		247 (116-401)	34 **** (20-84)	27 **** (18-33)
Albumin (35-50 g/L)		27 (25-29)	28 (25-38)	32 ** (28-37)
INR (0.9-1.2 ratio)		1.9 (1.7-2.3)	1.6 * (1.2-1.7)	1.3 **** (1.2-1.6)
Creatinine (45-120 µmol/L)		63 (50-127)	61 (48-72)	64 (52-78)
Sodium (135-145 mmol/L)		135 (132-137)	139 * (135-142)	133 (130-139)
Total white blood cell count ($4.0\text{-}11.0 \times 10^9$/L)		9.7 (6.7-17.4)	5.6 ** (3.9-8.7)	4.6 **** (2.9-7.3)
Neutrophil count ($2.2\text{-}6.3 \times 10^9$/L)		7.8 (4.8-16.3)	3.5 ** (2.2-6.3)	3.3 *** (2.2-5.2)
Lymphocyte count ($1.3\text{-}4 \times 10^9$/L)		1.2 (1.1-1.9)	1.2 (0.9-1.9)	1.0 (0.7-1.7)
Ascites (Median Child Pugh grade)		20 (65%) (2)	12 (75%) (2)	9 (60%) (3)
Hepatic encephalopathy (Median Child Pugh grade)		10 (32%) (1)	7 (44%) (1)	6 (40%) (1)
Child Pugh score (Grade A, B, C)		11 (0, 8, 23)	9 (3, 6, 7)	8 *** (4, 8, 3)
DF		53 (40-70)	NA	NA
MELD		25 (22-29)	14 **** (11-18)	12 **** (9-13)
Positive bacterial culture ◆		6 (19%)	7 (44%)	3 (20%)
Positive fungal culture ◆		8 (26%)	3 (19%)	0
1-month mortality		5/28 (18%) (3 patients excluded, data unknown)	2/16 (13%)	0
3-month mortality		6/26 (23%) (5 patients excluded, data unknown)	2/11 (18%) (5 patients excluded, data unknown)	0 (3 patients excluded, data unknown)
6-month mortality		9/24 (38%) (7 patients excluded, data unknown)	2/11 (18%) (5 patients excluded, data unknown)	1/9 (11%) (6 patients excluded, data unknown) •

*- compared to AAH, * - p<0.05, ** - p<0.01, *** - p<0.005, **** - p<0.0001

• - 1 patient transplanted

◆ - up to 6 months post-sampling

Abbreviations:
Acute Alcoholic Hepatitis: AAH; Alcohol-related cirrhosis active-drinkers: AA; Alcohol-related cirrhosis abstinent patients: ABA; International Normalised Ratio: INR; Discriminant function: DF; Model for End-stage Liver Disease: MELD

Table 3-2 Further clinical information on the ARC cohort

Group	Child Pugh Score (CPS)	Ascites (CPS)	Antibiotics	Variceal bleed
AA	12C	2	No	No
AA	9B	2	No	No
AA	8B	2	No	No
AA	10C	3	Yes	No
AA	12C	3	Yes	Yes
AA	7B	1	Yes	Yes
AA	5A	1	No	No
AA	8B	2	Yes	Yes
AA	13C	2	Yes	No
AA	5A	1	No	No
AA	10C	3	Yes	No
AA	5A	1	No	No
AA	9B	3	No	Yes
AA	12C	2	No	Yes
AA	10C	2	Yes	No
AA	8B	2	Yes	Yes
ABA	8B	1	No	No
ABA	6A	1	No	No
ABA	8B	3	No	No
ABA	6A	1	Yes	No
ABA	7B	1	No	No
ABA	5A	1	No	No
ABA	5A	1	No	No
ABA	9B	3	Yes	Yes
ABA	10C	3	Yes	No
ABA	7B	3	No	No
ABA	12C	2	No	No
ABA	8B	3	No	No
ABA	8B	3	No	No
ABA	8B	3	No	No
ABA	11C	3	Yes	No

Table 3-3 Bacterial and fungal isolates from patient cohorts

Group	Bacterial isolate	Fungal isolate
AAH	NA	Candida – urine
AAH	NA	Candida – ascites, urine
AAH	Vancomycin-resistant enterococcus – blood, ascites	Candida – ascites
AAH	Enterococcus faecium – blood Escherichia coli – urine	Candida – urine BAL - yeast
AAH	Escherichia coli – urine at 2 months Escherichia coli (ESBL) – blood at 3 months	NA
AAH	NA	Candida – urine at 1 month
AAH	Enterococcus faecium – blood, ascites Escherichia coli – ascites	Candida – ascites
AAH	NA	BAL - yeast
AAH	Enteroccus faecalis - ascites	NA
AAH	NA	Candida – urine
AA	Streptococcus parasanguinus - blood	Candida – ascites
AA	Staphylococcus aureus - wound	NA
AA	Enterococcus faecium – blood	NA
AA	Vancomycin-resistant enterococcus – ascites	Candida - sputum
AA	Enterococcus faecium – blood	Candida - sputum
AA	Staphylococcus aureus / mixed anaerobes - wound	NA
AA	Haemophilus influenza – sputum	NA
ABA	Staphylococcus aureus - wound	NA
ABA	Streptococcus gordonii - ascites	NA
ABA	Staphylococcus aureus - wound	NA

Abbreviations:
Bronchoalveolar lavage: BAL; Extended-Spectrum beta-lactamases; ESBL.

3.4.2 CD16 and CD11b expression

Baseline neutrophil surface receptor expression of the FcγRIII molecule CD16 and the β-integrin CD11b (C3bi) were determined amongst the patient groups and HC. CD16 expression was significantly reduced in AAH patients and ARC patients compared to HC ($p<0.05$) (Figure 3.2 and 3.3). There was no difference in the CD11b expression in patients compared to HC (Figure 3.4).

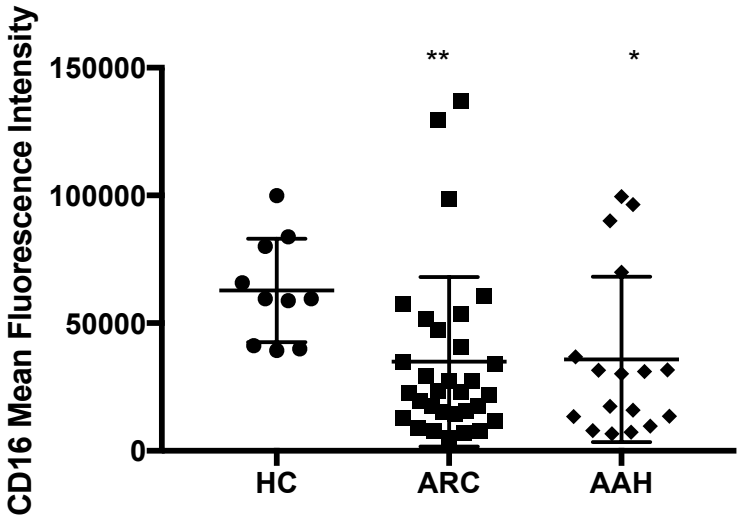


Figure 3.2 Decreased circulating neutrophil CD16 expression in patients with AAH and ARC compared to HC.

CD16 was significantly decreased in ARC and AAH compared to HC (* - compared to HC; ** - $p<0.01$, * - $p<0.05$). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

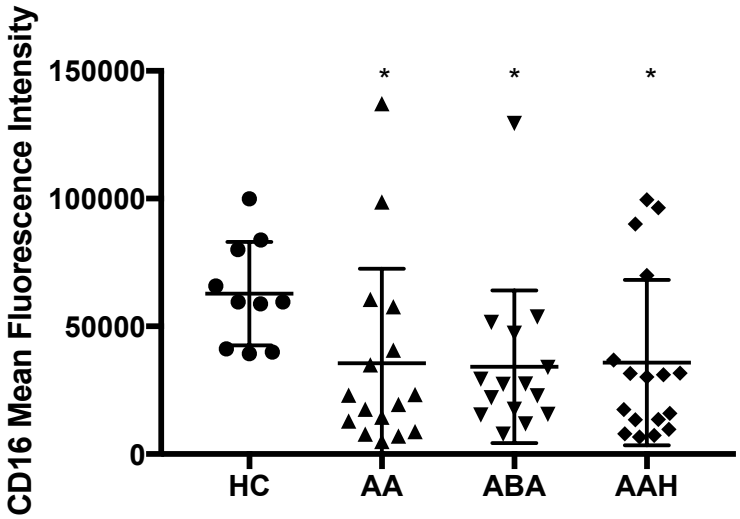


Figure 3.3 Decreased circulating neutrophil CD16 expression in patients with AAH and ARC divided into those actively drinking and those abstinent from alcohol.

CD16 was significantly decreased in AAH, AA and ABA compared to HC (* - compared to HC; * - $p<0.05$). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

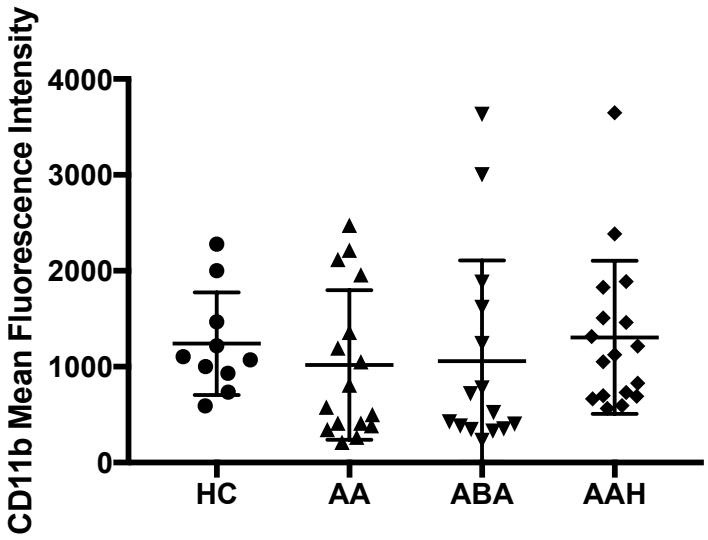


Figure 3.4 Circulating neutrophil CD11b receptor expression in AAH patients and ARC (divided into those actively drinking and abstinent) compared to HC.

There was no difference in the CD11b expression in patients compared to HC. Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

3.4.3 TLR2, 4 and 9 expression

Baseline neutrophil TLR2 expression was found to be significantly reduced in ARC patients compared to HC (p=0.01) and in AAH patients compared to HC (p=0.02) (Figure 3.5 and 3.6). Baseline TLR4 expression was significantly reduced in ARC patients compared to HC (p=0.006) and in AAH patients compared to HC (p=0.006) (Figure 3.7 and 3.8).

Baseline TLR9 expression was found to be significantly elevated in ARC patients compared to HC (p=0.04) and in AAH patients compared to HC (p=0.006) (Figure 3.9 and 3.10).

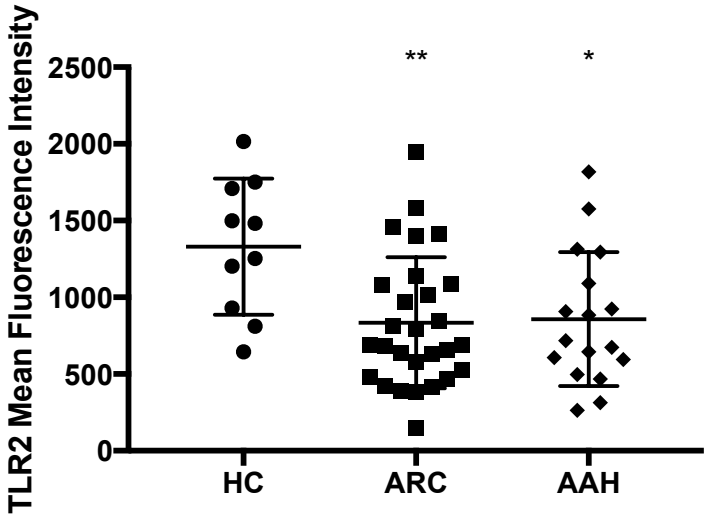


Figure 3.5 Decreased circulating neutrophil TLR2 expression in patients with AAH and ARC compared to HC.

TLR2 was significantly decreased in ARC and AAH compared to HC (* - compared to HC; ** - $p<0.01$, * - $p<0.05$). Ordinary one-way ANOVA with Tukey's multiple comparisons test were used to analyse the data (normal data).

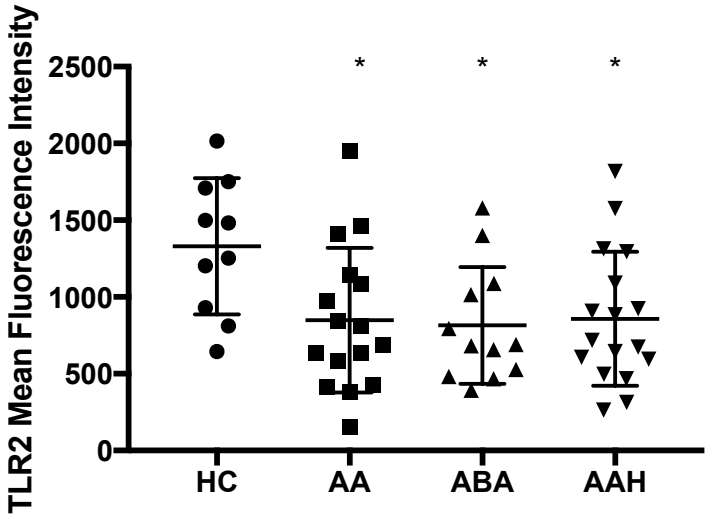


Figure 3.6 Decreased circulating neutrophil TLR2 expression in patients with AAH and ARC divided into those actively drinking and those abstinent from alcohol.

TLR2 expression was significantly decreased in AAH, AA and ABA compared to HC (* - compared to HC; * - $p<0.05$). Ordinary one-way ANOVA with Tukey's multiple comparisons test were used to analyse the data (normal data).

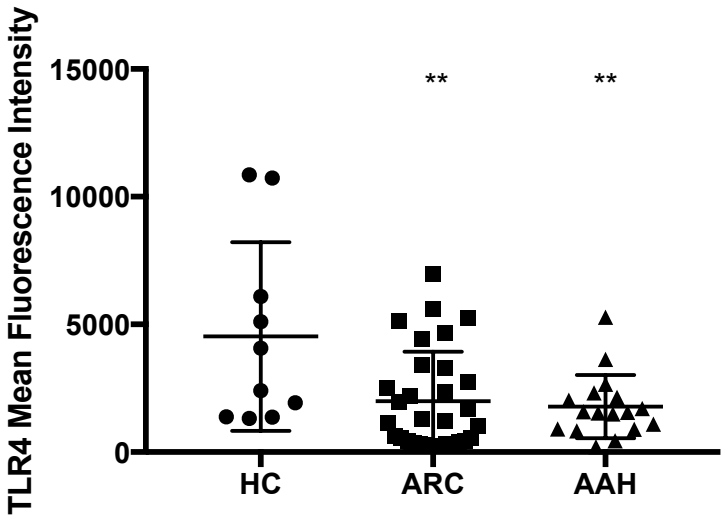


Figure 3.7 Decreased circulating neutrophil TLR4 expression in patients with AAH and ARC compared to HC.

TLR4 expression was significantly decreased in ARC and AAH compared to HC (* - compared to HC; ** - $p<0.01$). Ordinary one-way ANOVA with Tukey's multiple comparisons test were used to analyse the data (normal data).

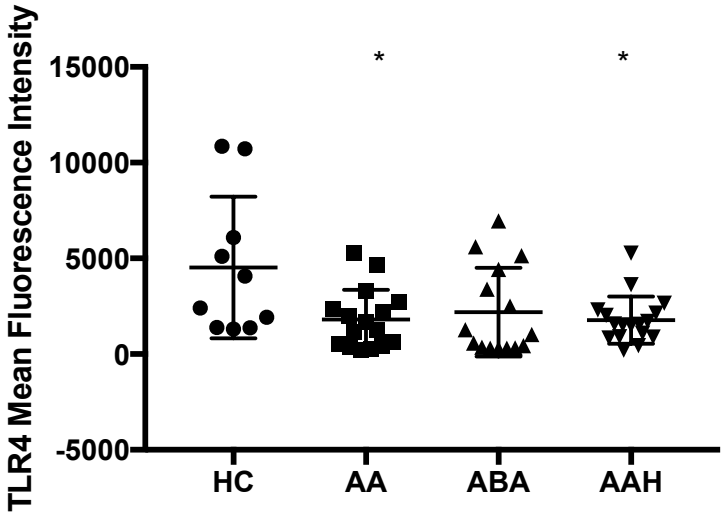


Figure 3.8 Decreased circulating neutrophil TLR4 expression in patients with AAH and ARC divided into those actively drinking and those abstinent from alcohol.

TLR4 expression was significantly decreased in AH and AA compared to HC (* - compared to HC; ** - $p<0.01$,* - $p<0.05$). Ordinary one-way ANOVA with Tukey's multiple comparisons test were used to analyse the data (normal data). The difference between ABA and HC did not reach significance using this test, $p=0.055$.

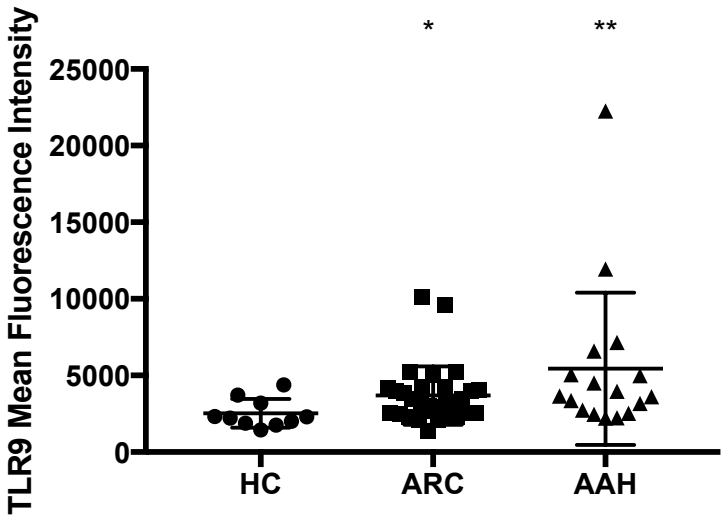


Figure 3.9 Increased circulating neutrophil TLR9 expression in patients with AAH and ARC compared to HC.

TLR9 expression was significantly increased in ARC and AAH compared to HC (* - compared to HC; ** - $p<0.01$,* - $p<0.05$). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

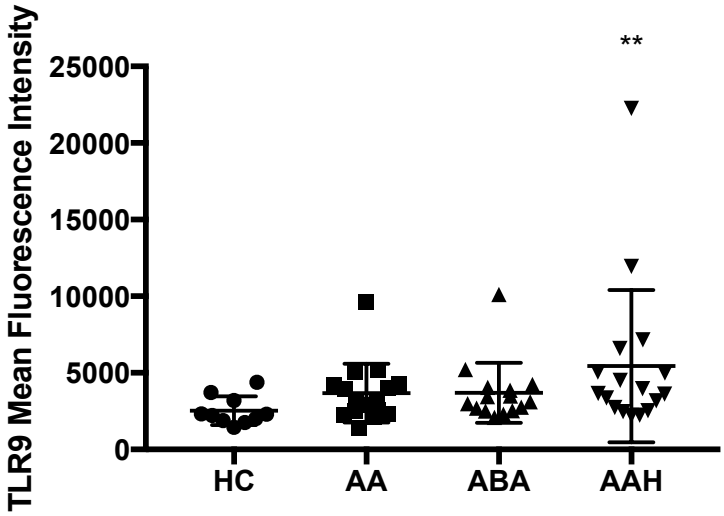


Figure 3.10 Increased circulating neutrophil TLR9 expression in patients with AAH compared to HC.

When ARC patients were divided into actively drinking and abstinent groups there was no significant difference between TLR9 expression in these groups compared to controls. TLR9 expression was significantly increased in AAH compared to HC (* - compared to HC; ** - $p<0.01$). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

3.4.4 Neutrophil phagocytosis

Phagocytic capacity was confirmed to be significantly reduced in the patient groups compared to HC (Figure 3.11 and 3.12). There was no significant difference seen between the AAH patients and those with ARC.

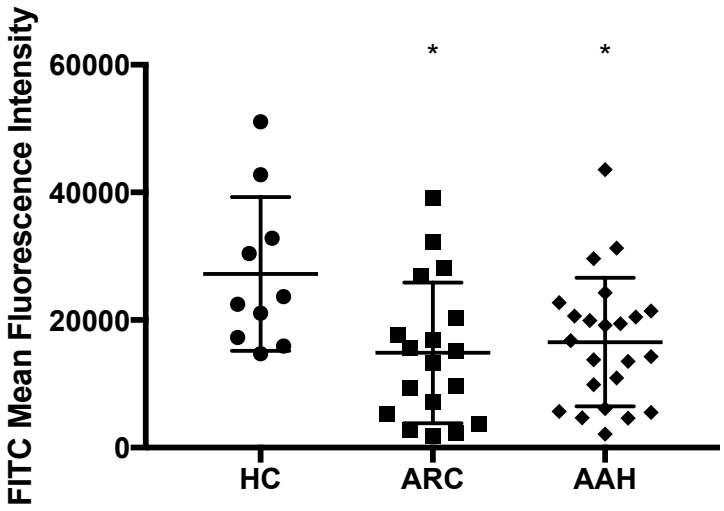


Figure 3.11 Impaired neutrophil phagocytic capacity in patients compared to HC.

Neutrophil phagocytic capacity expressed as FITC (labeled *E. coli*) mean fluorescence intensity was significantly reduced in both groups of patients compared to HC (* - compared to HC; * - $p<0.05$). Ordinary one-way ANOVA with Tukey's multiple comparisons test were used to analyse the data (normal data).

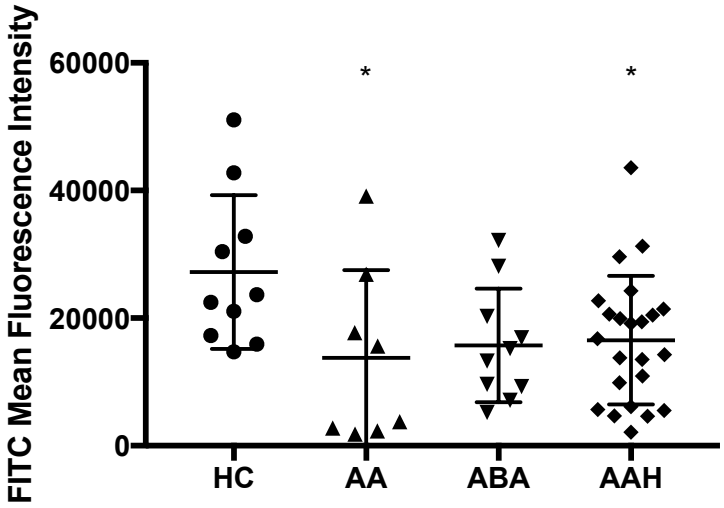


Figure 3.12 Impaired neutrophil phagocytic capacity in patients with AAH and ARC divided into those actively drinking and abstinent compared to HC.

Neutrophil phagocytic capacity expressed as FITC (labeled *E. coli*) mean fluorescence intensity (* - compared to HC; * - $p<0.05$). p value when HC compared to abstinent cirrhotic patients (ABA) did not reach significance at 0.058. Ordinary one-way ANOVA with Dunnett's multiple comparisons test were used to analyse the data (normal data).

3.4.5 Neutrophil oxidative burst

Resting burst was found to be significantly elevated in the AAH group compared to HC. The difference observed between HC and ARC, and ARC and AAH did not reach significance (Figures 3.13 and 3.14). There was significant variation within the groups and this is discussed further in the final chapter.

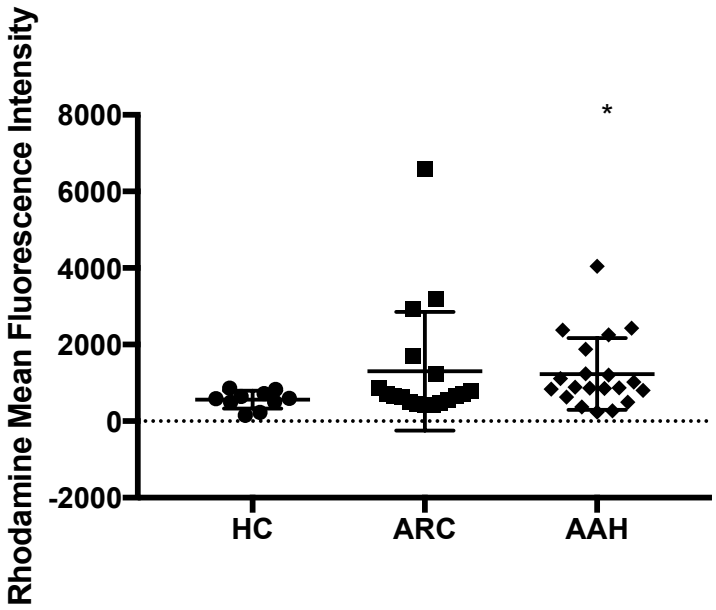


Figure 3.13 Resting neutrophil oxidative burst expressed as Rhodamine mean fluorescence intensity.

Resting neutrophil ROS production was found to be significantly higher in AAH patients compared to HC. (* - compared to HC; * - $p<0.05$, HC vs ARC $p=0.26$.) If the outlier within the ARC group is removed this does not significantly alter the statistical findings. Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

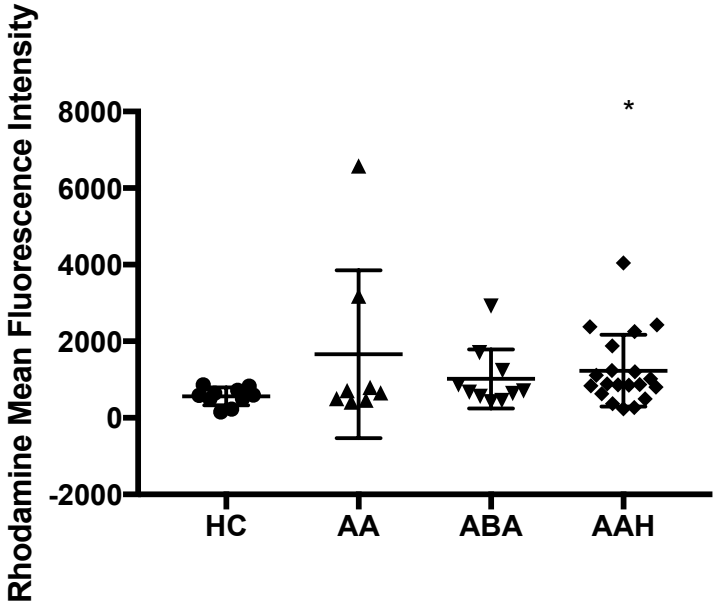


Figure 3.14 Resting neutrophil oxidative burst expressed as Rhodamine mean fluorescence intensity.

Resting neutrophil ROS production was found to be significantly higher in AAH patients compared to HC. (* - compared to HC; * - $p<0.05$). There was no significant difference seen in resting burst in the AA or ABA groups compared to HC. Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

On examination of low burst (fMLP stimulation), high burst (PMA stimulation) and phagoburst (*E coli* stimulation) (Methods 2.13.1), there was no significant difference seen between the groups.

3.4.6 Endotoxin

One of the distinguishing factors between HC, ARC and AAH patients was increasing plasma endotoxin. Plasma endotoxin levels were significantly elevated in AAH compared to HC ($p<0.001$) and to ARC ($p=0.024$); levels were also significantly higher in ARC compared to HC ($p=0.022$). Levels were found to correlate with severity of disease as defined by MELD in all patients ($r=0.520$; $p=0.018$) and Maddrey's discriminant function in the AAH cohort ($r=0.571$; $p=0.021$) (Figure 3.15).

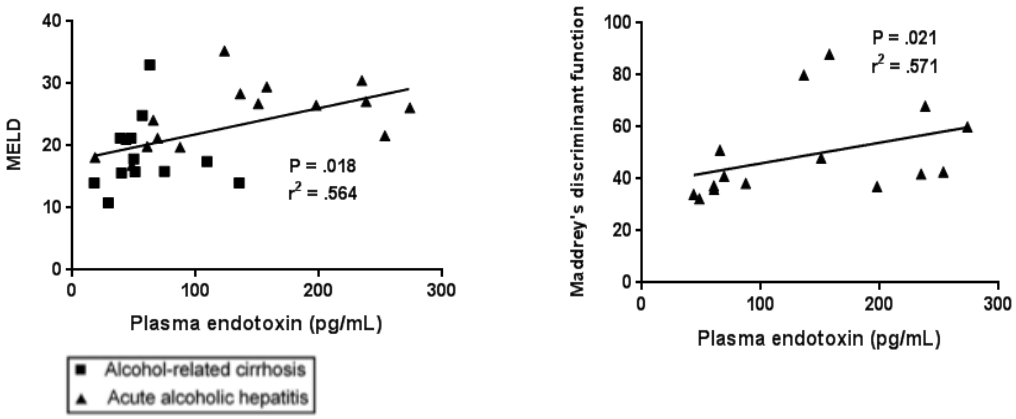


Figure 3.15 Plasma endotoxin levels were significantly elevated in patient groups compared to HC.

Endotoxin levels were found to correlate with disease severity as defined by MELD (r=0.520; p=0.018). Levels were significantly higher in AAH compared to ARC (p=0.024) and were found to correlate with disease severity as defined by Maddrey's discriminant function (r=0.571; p=0.021). Spearman's test was used to analyse the data.

3.4.7 LPS stimulation

Neutrophils from HC significantly up-regulated TLR-4 receptor expression in response to challenge with LPS but the cirrhotic and AAH patients failed to do so (Figure 3.16).

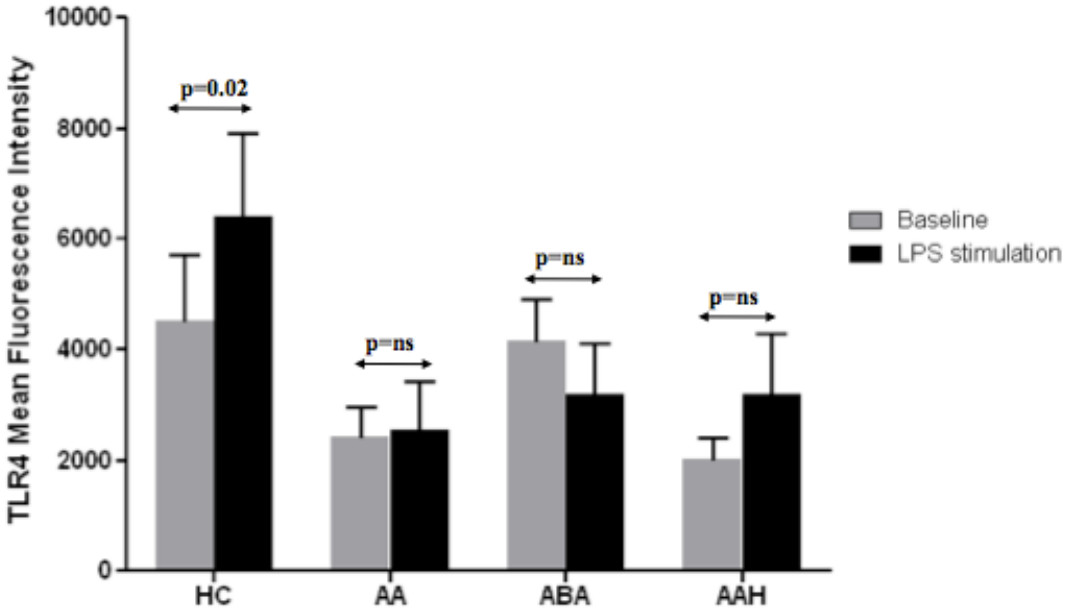


Figure 3.16 Neutrophil TLR4 expression pre- and post-LPS stimulation.

HC significantly up-regulated TLR4 in response to LPS stimulation (p=0.02); the patient groups did not significantly up-regulate TLR4 following LPS culture. Paired t-test was used to analyse the pre and post-LPS data (normal data).

3.4.8 Cytokine profiles

The most notable findings from the baseline plasma cytokine profiling were the differences seen in IL-6 and IL-8 levels. Plasma IL-8 levels were significantly higher in ARC compared to HC ($p=0.01$) and found to be of a significantly higher level in AAH compared to HC ($p<0.0001$). A significant difference was seen in AAH compared to ARC ($p<0.0001$) (Figure 3.17 and 3.18). Plasma IL-6 levels were significantly higher in ARC compared to HC ($p=0.0003$). Plasma IL-6 levels were significantly higher in AAH compared to HC ($p<0.0001$). Unlike circulating plasma IL-8 levels there was no difference in IL-6 when ARC and AAH patient groups were compared (Figure 3.19 and 3.20).

G-CSF, IL-10 and TNF-α were not detected in the baseline plasma AAH samples.

Following 2 hour stimulation with LPS, plasma supernatant IL-8 and IL-6 levels were found to significantly increase (in order of 100-fold or greater) in the HCs. In the AAH cohort although basal levels of IL-8 and IL-6 were significantly higher, no such increase was seen. High levels appear to be observed at basal state perhaps relating to circulating endotoxin in this group, with subsequent lack of response on additional LPS challenge.

Figures 3.21 and 3.22 represent the fold-change in IL-8 and IL-6 pre- and post-culture with LPS.

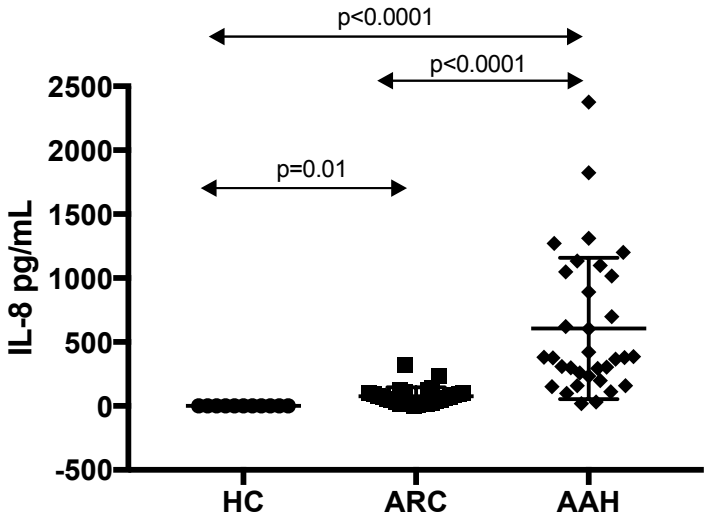


Figure 3.17 Increased circulating IL-8 levels in patients with AAH and ARC compared to HC.

Plasma IL-8 (pg/mL) measured using CBA was significantly increased in ARC compared to HC (p=0.01). IL-8 levels were found to be significantly elevated in AAH compared to HC (p<0.0001) and ARC (p<0.0001). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

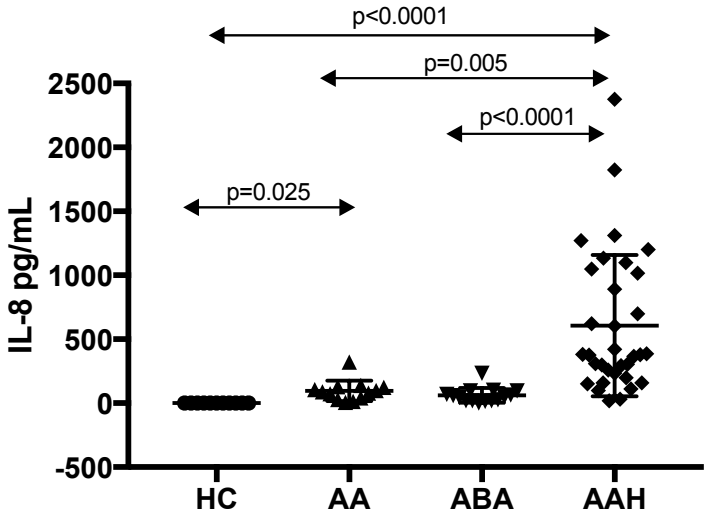


Figure 3.18 Increased circulating IL-8 levels in patients with AAH and actively drinking ARC patients compared to HC.

Plasma IL-8 (pg/mL) was significantly increased in AA compared to HC (p=0.025). IL-8 levels were found to be significantly elevated in AAH compared to HC (p<0.0001) and compared to AA (p=0.005) and ABA groups (p<0.0001). The difference between the abstinent ARC patients and HC did not reach significance when patients were divided. Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

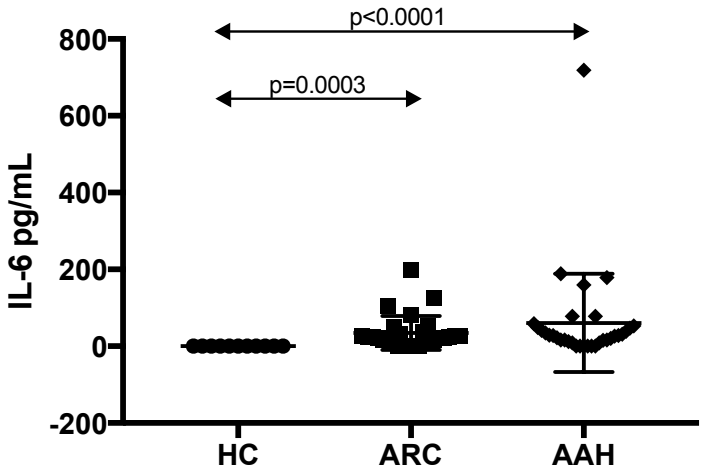


Figure 3.19 Increased circulating IL-6 levels in patients with AAH and ARC compared to HC.

Plasma IL-6 (pg/mL) measured using CBA was significantly increased in ARC compared to HC (p=0.0003). IL-8 levels were found to be significantly elevated in AAH compared to HC (p<0.0001). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

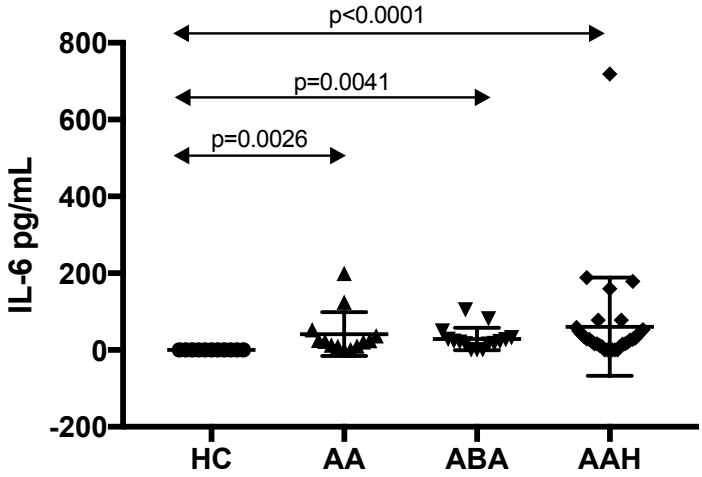


Figure 3.20 Increased circulating IL-6 levels in patients with AAH and actively drinking and abstinent ARC patients compared to HC.

Plasma IL-6 (pg/mL) was significantly increased in AA compared to HC (p=0.0026), levels were also significantly increased in ABA compared to HC (p=0.0041) and in AAH compared to HC (p<0.0001). There were no significant differences seen between the patient groups. Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

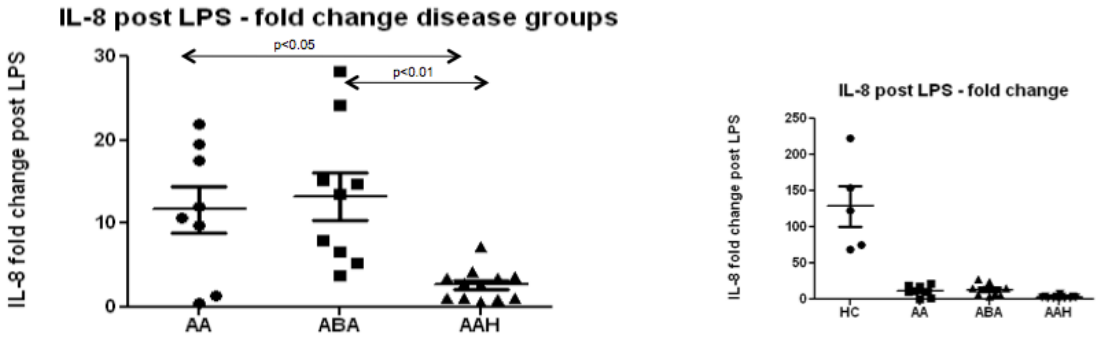


Figure 3.21 IL-8 fold change pre- and post-LPS stimulation in patient groups (left) and patient groups and HC (right).

The fold change was significantly lower in the AAH group compared to actively drinking cirrhotic patients ($p<0.05$) and compared to abstinent cirrhotic patients ($p<0.01$). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

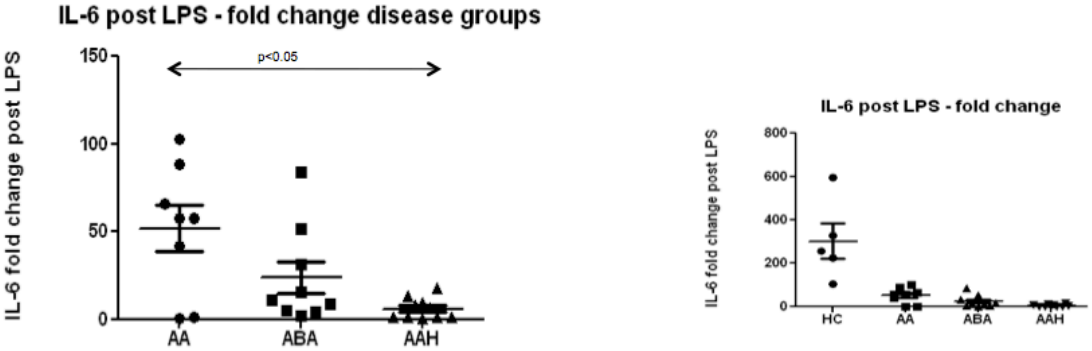


Figure 3.22 IL-6 fold change pre- and post-LPS stimulation in patient groups (left) and patient groups and HC (right).

The fold change was significantly lower in the AAH group compared to actively drinking cirrhotic patients ($p<0.05$). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

3.4.9 Lactoferrin

Plasma lactoferrin, released from secondary granules in neutrophils, was significantly increased in the AAH patients compared to cirrhotics without AAH ($p<0.005$) and HC ($p<0.0001$) (Figure 3.23). There was a positive correlation between plasma lactoferrin and plasma IL-8 in AAH patients ($r=0.74$) (Figure 3.24).

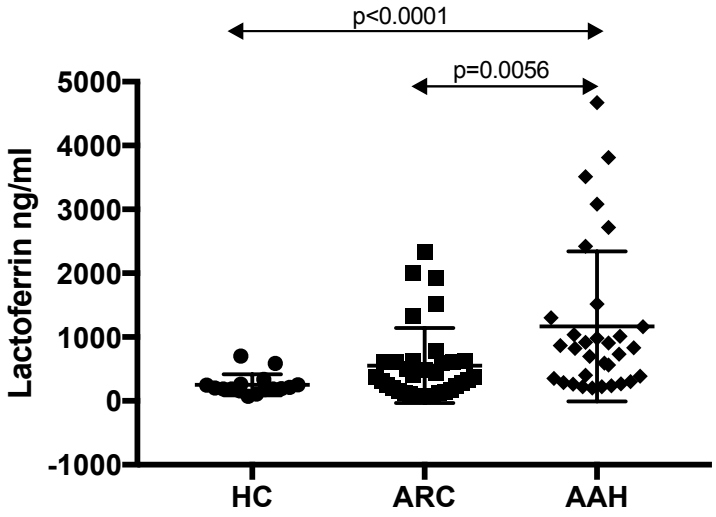


Figure 3.23 Increased circulating lactoferrin levels in AAH compared to ARC and HC.

Lactoferrin was significantly increased in the AAH patients compared to cirrhotics without AAH ($p<0.005$) and HC ($p<0.0001$). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

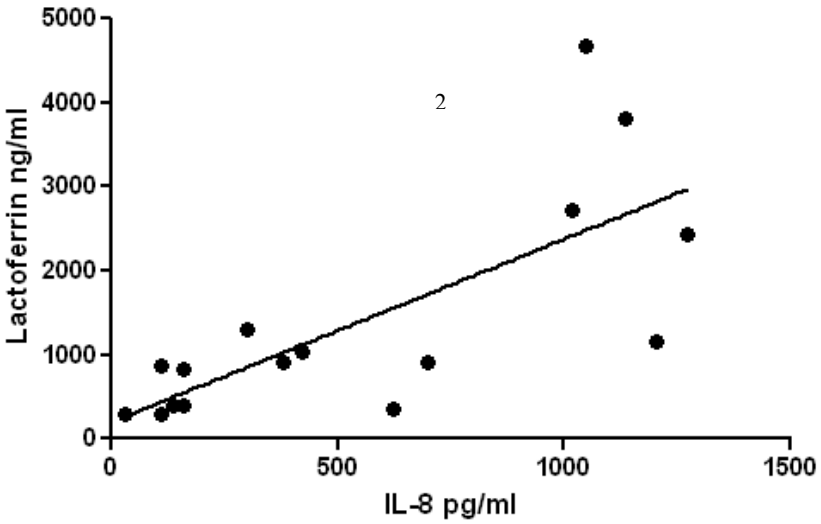


Figure 3.24 Correlation between plasma lactoferrin and IL-8 in AAH.

There was a direct correlation between baseline plasma lactoferrin and IL-8 in AAH (r=0.74).

3.4.10 Endothelial markers

Circulating VCAM-1 levels were decreased in AAH patients compared to HC ($p<0.0001$) and ARC patients ($p<0.0005$) (Figure 3.25). Circulating ICAM-1 levels were decreased in AAH patients compared to ARC patients ($p=0.03$) (Figure 3.26). Circulating E-selectin was significantly higher in AAH patients compared to HC ($p=0.002$), levels were also significantly higher in ARC patients compared to HC ($p=0.013$); there was no significant difference between ARC and AAH (Figure 3.27). Circulating L-selectin levels were significantly higher in ARC patients compared to HC ($p=0.023$), levels were also found to be higher in ARC compared to AAH patients ($p=0.014$); there was no difference between AAH and HC (Figure 3.28). There was no significant difference in VEGF between the groups (Figure 3.29).

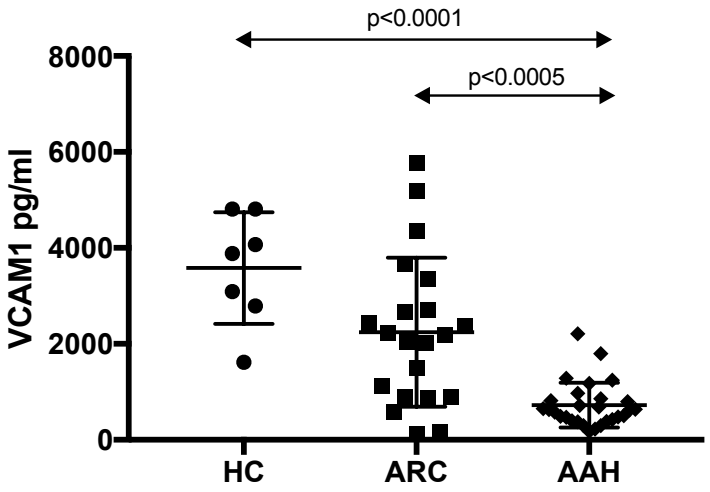


Figure 3.25 Circulating VCAM-1 levels in patients and HC.

Circulating VCAM-1 levels were decreased in AAH patients compared to HC ($p<0.0001$) and ARC patients ($p<0.0005$). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

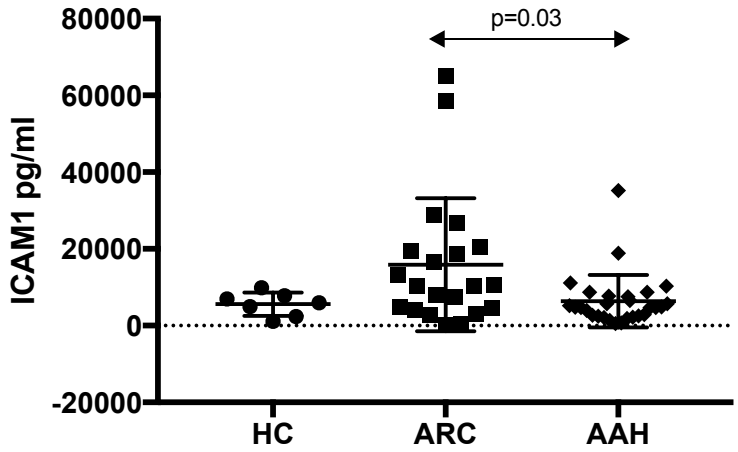


Figure 3.26 Circulating ICAM-1 levels in patients and HC.

Circulating ICAM-1 levels were decreased in AAH patients compared to ARC patients (p=0.03). Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

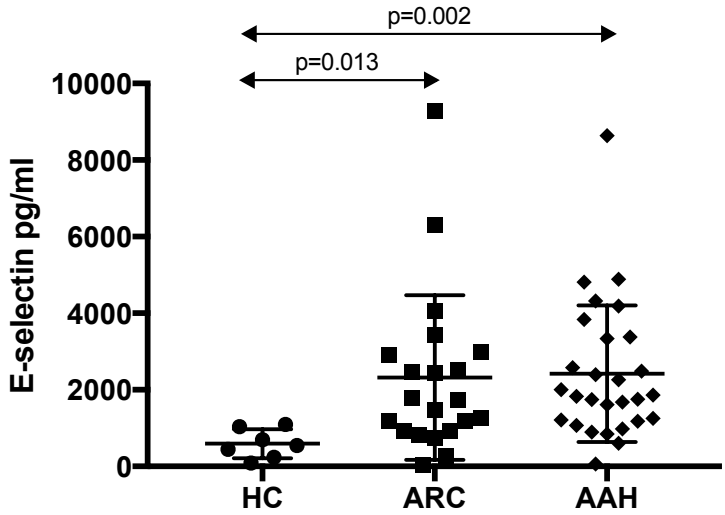


Figure 3.27 Circulating E-selectin levels in patients and HC.

E-selectin was significantly higher in AAH patients compared to HC (p=0.002), levels in ARC patients were significantly higher compared to HC (p=0.013).
Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

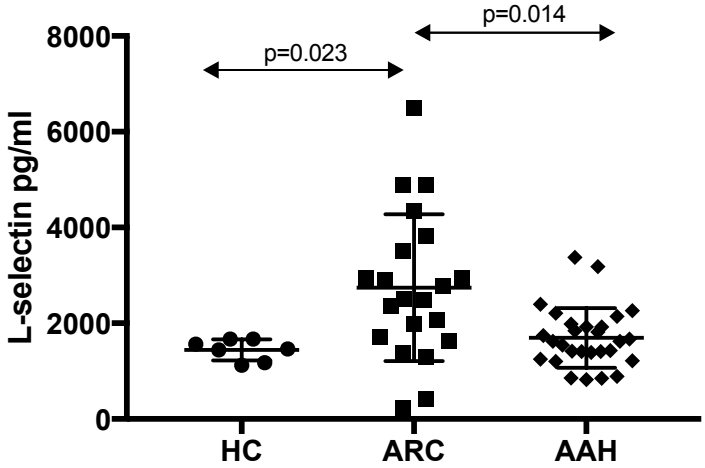


Figure 3.28 Circulating L-selectin levels in patients and HC.

Circulating L-selectin levels were significantly higher in ARC patients compared to HC (p=0.023), levels were also found to be higher in ARC compared to AAH patients (p=0.014), there was no difference between AAH and HC. Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

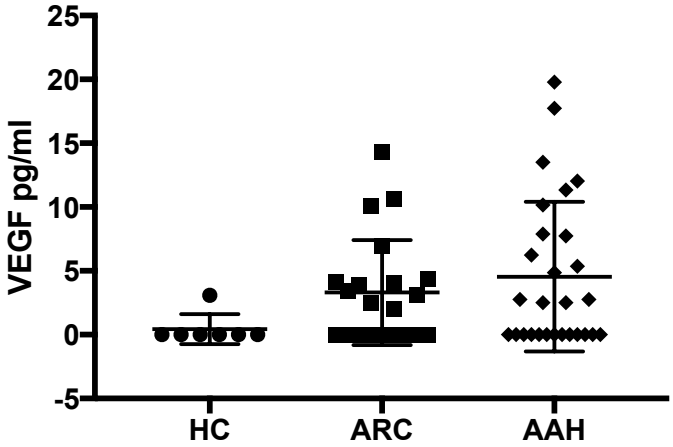


Figure 3.29 Circulating VEGF levels in patients and HC.

There were no significant differences in soluble VEGF observed between groups. Kruskal-Wallis test with Dunn's multiple comparisons test were used to analyse the data.

3.4.11 Effect of PD1/TIM3 blockade on neutrophil function

This work was done in collaboration with Lee Markwick at the Foundation for Liver Research [78]. A marked increase in the expression of PD1 (p=0.036) and galectin-9 (p=0.36) was found on CD4 T cells in AAH compared with healthy controls. On CD8 T cells, increased expression of PDL1 (p=0.041), TIM3 (p=0.042), and galectin-9 (p=0.037) were also observed. This was not seen in ARC. In AAH, but not in ARC, an increase in the frequency of LPS-challenged PBMC producing IL-10 (p=0.020) was found compared with HC. Conversely, a marked decrease in the frequency of LPS-challenged PBMC producing IFN gamma (p=0.001) was found. Expressed as a ratio (IFN gamma/IL-10), there was a skew in the LPS-mediated immune response in AAH compared with patients with ARC (p=0.026) and HC (p=0.004), demonstrating that the antibacterial immunity of PBMCs is skewed in AAH toward an immunosuppressive IL-10-dominated response, perhaps reflective of a CARS predominant phase. Concentrations of endotoxin observed in AAH were found to induce hyperexpression of PD1 and TIM3 on HC Tregs, CD4 and CD8 cells. In AAH, inhibition of PD1/TIM3 increased the frequency of bacterially challenged PBMC producing IFN gamma (p=0.048) and reduced IL-10-producing PBMCs (p=0.002). In AAH, *ex vivo* treatment of whole blood with anti–PD1 and anti–TIM3 blocking antibodies enhanced neutrophil phagocytosis/ingestion capacity (p=0.005) (Figure 3.30). Spontaneous oxidative burst remained unchanged and the production of IL-1β, IL-6, IL-8 and TNF-α was not provoked by treatment.

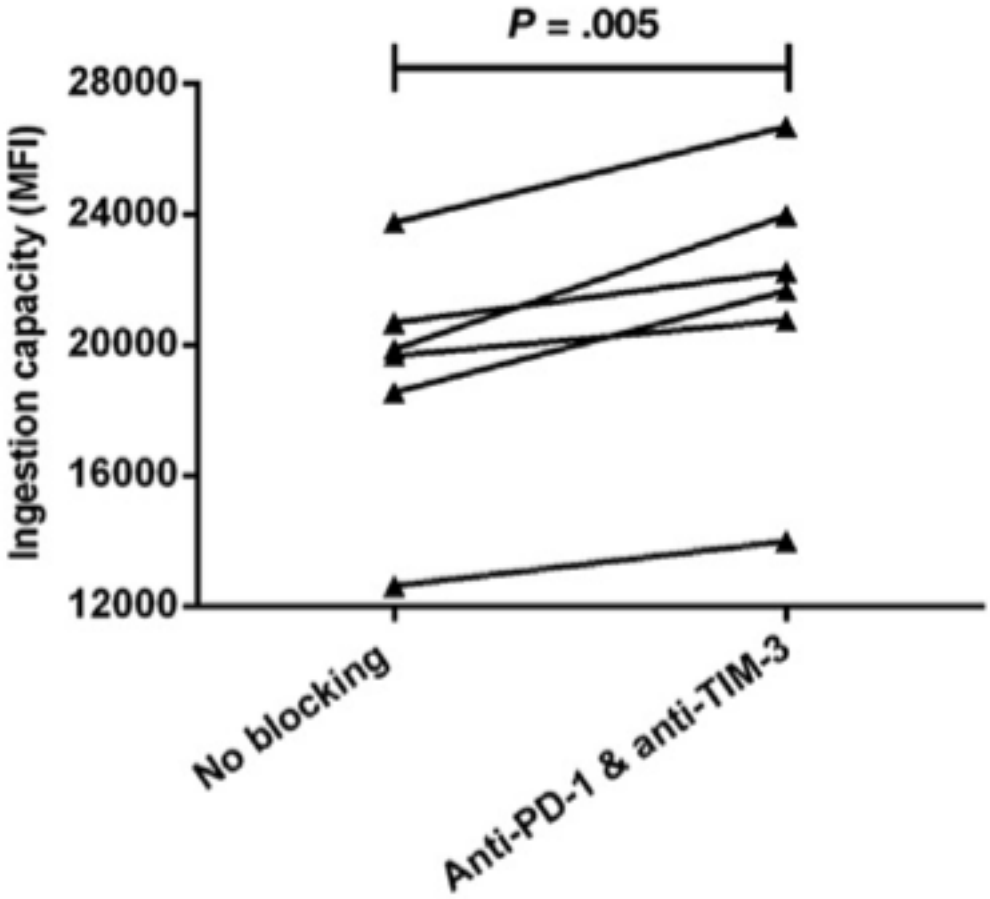


Figure 3.30 Neutrophil phagocytosis pre- and post- blockade of PD-1 and TIM-3.

Ingestion capacity was significantly increased following treatment with anti-PD1 and anti-TIM3 blocking antibodies.

3.5 Discussion

In this study neutrophils from AAH patients were confirmed to be activated with evidence of increased production of ROS and lactoferrin release. Resting burst was only found to be significantly elevated in the AAH group; this is in keeping with previously published data [154]. Interestingly, a significant difference was observed in circulating lactoferrin levels between the AAH and ARC groups. Plasma IL-8, a potent neutrophil-attracting chemokine, was significantly increased in AAH patients compared to HC and cirrhotics as previously seen in multiple studies [208]. IL-8 was found to directly correlate with plasma lactoferrin. Lactoferrin levels did not correlate with disease severity, as defined by Maddrey's discriminant function; sequential analyses and outcome data are discussed further in chapter 4.

Liver biopsies were not routinely performed to confirm the diagnosis of AAH and, accepting that the issue of biopsy in clinical practice in AAH remains contentious, the benefits of doing this within this study would have been numerous. It is possible that some patients with ARC did have superimposed AAH and histological specimens would have provided additional important research material. The patients were, however, screened carefully and the clinical inclusion and exclusion criteria for the STOPAH study were applied.

It would be interesting to study neutrophils that hone to the liver in AAH and we do not know if the findings on the peripherally circulating neutrophils are replicated on those within the liver. This would be valuable to explore but would require fresh liver tissue samples; portal vein neutrophil analyses could also be

compared to peripheral samples and cross-liver sampling during hepatic venous pressure gradient studies could be performed. In addition, further work into NET formation in AAH may be of value. In recent work in mice it has been shown that after MRSA infection, bacteria are captured by Kupffer cells residing in the liver, subsequently recruited neutrophils release NETs into the vasculature in an attempt to eradicate the pathogen. It was found that the majority of the liver injury was induced by the NETs and when NET production was blocked, damage was ameliorated [144]. Interestingly, lactoferrin is also believed to be one of the components of NETs and is thought to inhibit NET formation [209]. Perhaps the release of lactoferrin from peripherally circulating neutrophils, responsible for the high plasma levels observed in my work, allows the persistence of NETs within the liver in AAH as honing neutrophils may be functionally deplete in this protein. We have shown that neutrophil granule release is dysregulated in ARC [210], this may be of even greater importance in AAH. Further work on liver tissue, including neutrophil elastase and myeloperoxidase assessment, may allow this to be elaborated upon. Strategies that inhibit NET production or induce the shedding of anchor molecules, such as von Willebrand Factor, might prevent the ongoing tissue damage seen in AAH and other liver conditions. The trigger of AAH, and particularly whether there is an infective trigger that precipitates the cascade of damage also warrants further exploration. This concept, I believe, is further supported by the finding that >90% of AAH patients in Vergis *et al's* study had detectable bacterial DNA levels, a rate substantially higher than seen in HCs, patients with bloodstream infections, and patients with other forms of decompensated liver disease [97].

High circulating endotoxin levels were confirmed in the patient groups studied; these were of a greater level in AAH and were found to correlate with disease severity. Neutrophil antibacterial activities and key detecting receptors (TLRs) were found to be dysfunctional in the patient groups. Across the entire spectrum of patients there appeared to be a blunted response to LPS. A possible explanation for this may be chronic sub-clinical exposure to pathogen secondary to gut bacterial translocation in cirrhosis, which leads to a dampened response when a significant bacterial insult ensues. This finding does not appear to be specific to AAH and may have wider implications for cirrhosis of all causes. Gut modulation remains a key focus of investigation and our laboratory have further studies underway examining the effect of the non-absorbable antibiotic rifaximin on neutrophil parameters. Unfortunately, it was not possible to meaningfully correlate endotoxin with neutrophil TLR4 results due to the small numbers of patients with matched analyses and this would have been interesting to evaluate along with sequential endotoxin analyses in the entire group of patients.

Only patients with ALD were studied and this was because the group of interest were those with AAH and the ARC / HCs were used as comparators. It would have added to the study to have included patients with cirrhosis of other aetiology. In Taylor's study aetiology of liver disease did not impact on neutrophil stimulated burst or phagocytosis, which was seen to decline with worsening severity of liver disease; alcohol was the most common cause of cirrhosis in these patients [152], mimicking a 'real world' cohort of cirrhotic patients.

The influence of active alcohol use did not appear to impact on neutrophil phenotype. Phagocytosis however was not as severely dampened in the ABA group. This may, in fact, be explained by the difference in Child-Pugh scores and severity of liver disease as this was lower in the ABA compared to the AA group. It is difficult to comprehensively examine the impact of active alcohol use in these studies as there are many confounding factors, and patients need to be matched as closely as possible to draw any accurate conclusions. One of the drawbacks of the study is the heterogeneity of the patient cohorts and this, to some extent, is unavoidable in observational human studies such as mine. With increased patient numbers it would have been of use to further divide the ARC groups, again it has to be noted that these were a heterogeneous group. The HC also tended to be younger, it would have added to the work if these subjects had been more closely matched. In addition, the study of excess alcohol drinkers without evidence of liver disease or early fatty change would have provided further information on the direct impact of alcohol. In fact, future studies should include this group with large numbers so that the precipitants and disease triggers can be understood, this is discussed further in my final chapter.

In contrast to neutrophil TLR2 and TLR4 expression, TLR9 was found to be significantly increased in the AAH group. This is out of keeping with Stadlbauer's data where TLR2, TLR4 and TLR9 were all found to be elevated [155]. This may, in part, be explained by the different experimental techniques used; in Stadlbauer's study normal neutrophils were incubated with plasma from patients with AAH. The reduced TLR2 and TLR4 expression is likely to culminate in reduced antibacterial surveillance, but why the neutrophils have reduced

expression is not explained. The elevated intracellular TLR9 expression may, on the other hand, be induced by high circulating DAMPS secondary to hepatocellular damage in AAH. In paracetamol-induced acute liver failure neutrophil TLR9 has been found to correlate with ammonia levels [211]. Ammonia levels were not routinely checked on the patients I studied and, in retrospect, the relationship between ammonia and neutrophil TLR9 would have been interesting to confirm in my patient cohorts, as would the effect on neutrophil TLR9 following plasma exposure to various stimulants including ammonia. To some extent, time constraints limited further evaluation of TLR9 and this, within the paradigm of sterile inflammation, would be an interesting area of study.

When soluble endothelial markers were examined, E-selectin was found to be elevated across the spectrum of patients recruited, although levels tended to be higher in AAH there was no significant difference between the AAH and ARC groups. L-selectin was only found to be increased in the ARC group. Perhaps surprisingly, VCAM1 and ICAM1 were reduced in AAH compared to ARC, it is important to highlight that tissue levels or expression were not examined and the temporal relationship of when patients present to hospital may, as with any interpretation, be of relevance here. It would have been of interest to examine the AAH neutrophils for chemokine receptors and cell-adhesion molecules such a CD181 and P-selectin glycoprotein ligand-1 (PSGL-1), and future studies should focus on neutrophil-endothelial interaction to strengthen the understanding of the process of migration of neutrophils to the liver parenchyma.

One of the most important findings demonstrated is the potential for immune dysfunction reversibility in this condition. The indirect improvement of PD1/TIM3 blockade on neutrophil antibacterial responses in AAH through improved interferon gamma/IL-10 responses is of particular interest and reveals a previously unknown potential therapeutic target. Importantly this strategy did not aggravate factors associated with the inflammatory state in AAH. The immunosuppression rather than the initial cytokine storm may be the predominant driver of mortality in this group of patients and greater understanding of why certain patients appear to enter a harmful compensatory anti-inflammatory response syndrome [107] is needed. The evolution of the disease state of AAH and impact of current therapies in use forms the basis of the next chapter.

4 Longitudinal analyses of neutrophil function and effect of current therapies on innate immune function in acute alcoholic hepatitis

4.1 Background

It is increasingly recognised that patients with AAH have a dynamic disease course; better understanding of this may help tailor specific therapies to individual patients with reduction in morbidity and mortality.

No study has longitudinally investigated changes in immune function in this condition and the impact, at this level, of current treatments used. A particular question of interest is why certain individuals with AAH enter a state of immune exhaustion; increased examination of this aspect with longitudinal studies would add to our understanding of this potentially harmful phase of illness.

4.2 Aim of investigation

To prospectively examine neutrophil and plasma cytokine profiles (as described in chapter 3) in the AAH cohort through sequential analyses. The majority of patients were enrolled in the STOPAH trial which enabled observation of the natural disease course in addition to the effect of current AAH therapies on innate immune function. The STOPAH study, previously described, randomised patients with a clinical diagnosis of AAH to one of four groups; one receiving pentoxifylline-matched placebo and prednisolone-matched placebo, a second group receiving 40mg of prednisolone daily and a pentoxifylline-matched placebo, the third group receiving 400mg of pentoxifylline three times daily and prednisolone-matched placebo, and the fourth group receiving 40mg of prednisolone daily and 400mg of pentoxifylline three times daily. Pentoxifylline was not found to improve survival in patients with alcoholic hepatitis. Prednisolone was associated with a reduction in 28-day mortality that did not

reach significance, with no improvement in outcomes at 90 days or 1 year [98]. For the purposes of my analyses my patient cohort were divided into those who received prednisolone and those who did not.

4.3 Methods

4.3.1 Patients and study design

A prospective cohort study was performed on patients presenting with severe AAH as previously defined. Blood samples were collected within 48 hours of hospital admission, prior to administration of any immunosuppressant therapy. Follow-up analyses were performed on day 7 and day 28 when possible (day 1 pertains to the day of sampling). Eight patients had analyses at later time points.

4.3.2 Experiments

Neutrophil phenotype, function, characterization post-LPS stimulation, plasma lactoferrin, cytokine and endothelial marker analyses were performed as previously described.

4.4 Results

4.4.1 Clinical data

Table 3.1 outlines the clinical demographics of the cohorts studied. The median age of the AAH group was 49 (IQR 34-60), the median DF was 53 (IQR 40-70) and the median MELD was 25 (IQR 22-29). Table 4.1 details the mortality and incidence of infection, defined as a positive culture over a 6-month follow-up period. Given the STOPAH study findings, for my analyses patients were reviewed as a cohort and subsequently divided into two groups – those who received prednisolone and those who did not. Twenty-eight day mortality for the

group of patients studied was 18%. Ten out of 28 (36%) developed culture positive infection over the 6-month follow-up. Eleven out of 26 patients received prednisolone therapy (treatment data not available in 5 patients). There was no significant difference in DF or MELD between the patients who received prednisolone and those who did not. Twenty-eight day mortality (taken from the time of sampling) in the prednisolone-treated group and the non-prednisolone-treated group was 18% and 20%, respectively. A higher proportion of prednisolone-treated patients developed culture-positive infection (2 patients in this arm were removed from results due to unavailable data over the defined follow-up period).

Table 4-1 Mortality at 28 days, three- and six-months and culture positive infection incidence in the AAH patients studied

	Whole group	Prednisolone-treated	Non-prednisolone treated
28 day mortality	5/28 18% (3 patients excluded, data unknown)	2/11 18%	3/15 20%
3-month mortality	6/26 23% (5 patients excluded, data unknown)	3/9 33% (2 patients excluded, data unknown)	3/15 20%
6-month mortality	9/24 38% (7 patients excluded, data unknown)	6/7 86% (4 patients excluded, data unknown)	3/15 20%
Culture positive; 6-month follow-up	10/28 36% (3 patients excluded, data unknown)	6/9 67% (2 patients excluded, data unknown)	4/15 27%

4.4.2 Neutrophil function and outcome

No significant difference was observed in phagocytic capacity or resting burst when the 6-month survivors and non-survivors were compared. 6-month outcome was used as numbers were too small to run statistics with regard to outcome at earlier time points. There was no significant difference in phagocytic capacity between the culture-positive and culture-negative patients (Figure 4.1). On analyses of the sub-group of patients treated with prednisolone, again no difference in phagocytosis was seen between those who developed culture positive infection and those who did not (Figure 4.2). No significant difference was seen in resting burst between the culture-positive and culture-negative patients.

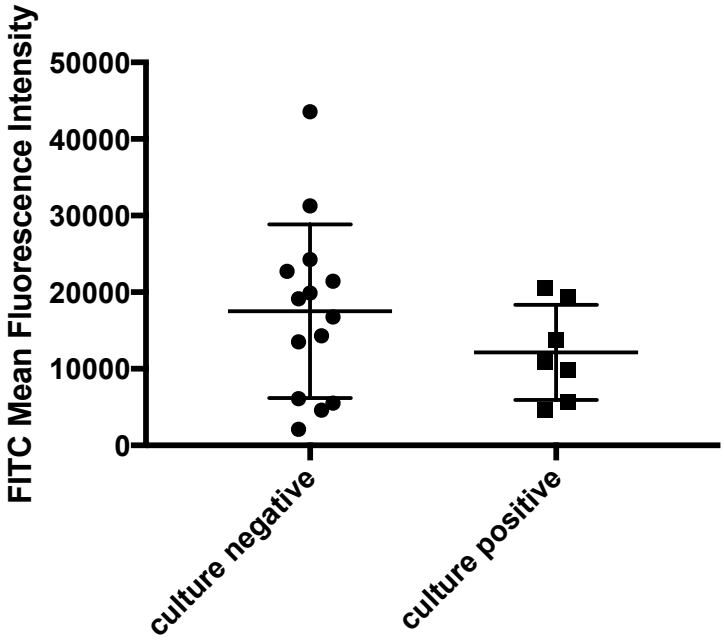


Figure 4.1 Phagocytic capacity in the culture-negative and culture-positive AAH patients.

No significant difference was seen between the 2 groups. Unpaired t-test was used to analyse the data (normal data).

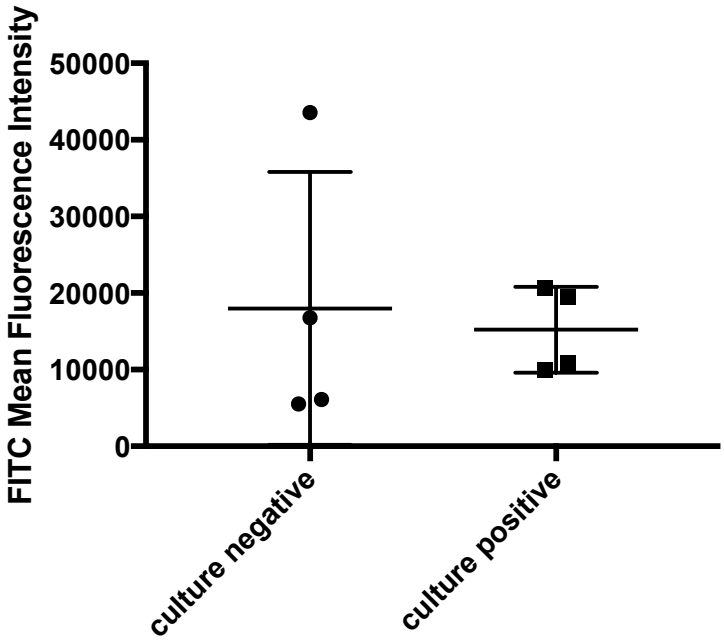


Figure 4.2 Phagocytic capacity in the culture-negative and culture-positive AAH sub-group of patients treated with prednisolone.

The TLR4 fold change post-LPS stimulation was significantly lower amongst the 6-month non-survivors compared to the 6-month survivors ($p=0.02$) (Figure 4.3).

There was no significant difference in TLR4 fold change post-LPS between the patients who developed culture-positive infection over the 6-month follow-up and those who did not (Figure 4.4).

Numbers were not large enough to perform sub-group analyses on the prednisolone-exposed patients.

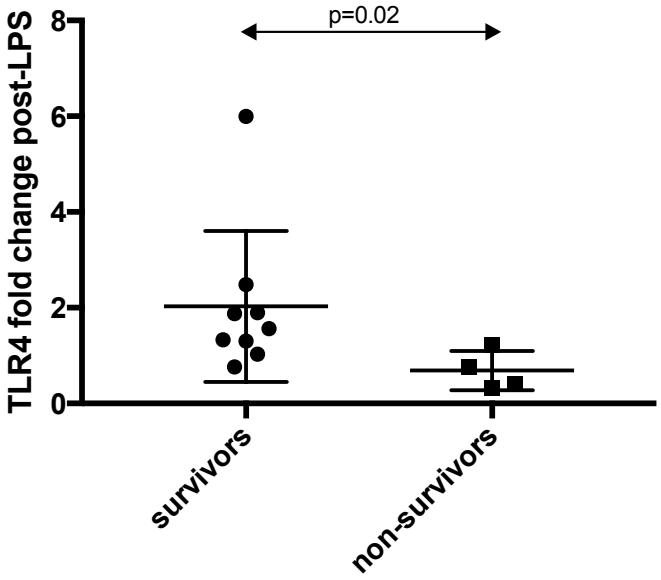


Figure 4.3 TLR4 fold change post-LPS challenge in the AAH survivors and non-survivors.

The fold change was significantly lower in the 6-month non-survivors (p=0.02). Mann-Whitney test was used to analyse the data.

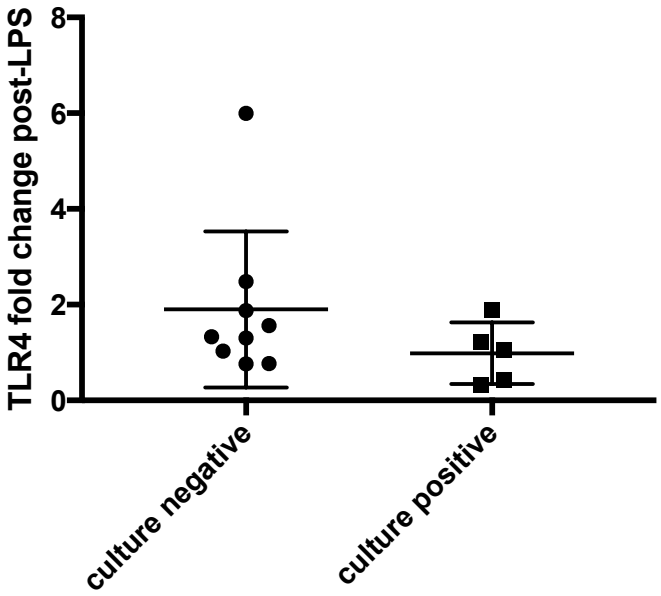


Figure 4.4 TLR4 fold change post-LPS challenge in the culture-positive and culture-negative AAH patients.

Mann-Whitney test was used to analyse the data.

4.4.3 Lactoferrin, IL-8 and outcome

There was no difference in baseline plasma lactoferrin when 6-month AAH survivors and non-survivors were compared. Similarly, there was no difference in lactoferrin levels between the group that developed culture-positive infection and the group that did not. There was no difference in baseline plasma IL-8 levels when the 6-month survivors were compared to the non-survivors (Figure 4.5). Although the IL-8 levels tended to be higher in the AAH patients who went on to develop culture-positive infection compared to those who did not, there was no significant difference detected between the two groups ($p=0.1$) (Figure 4.6).

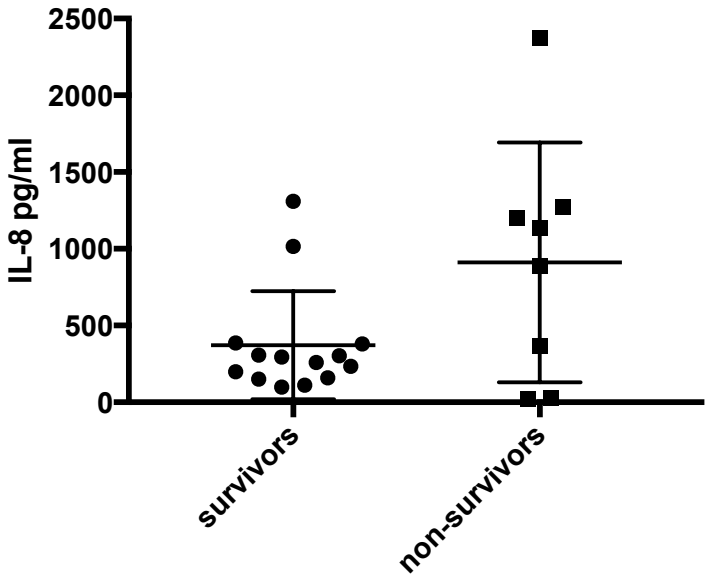


Figure 4.5 Baseline plasma IL-8 levels in the AAH 6-month survivors and non-survivors.

There was no significant difference between the 2 groups. Mann-Whitney test was used to analyse the data.

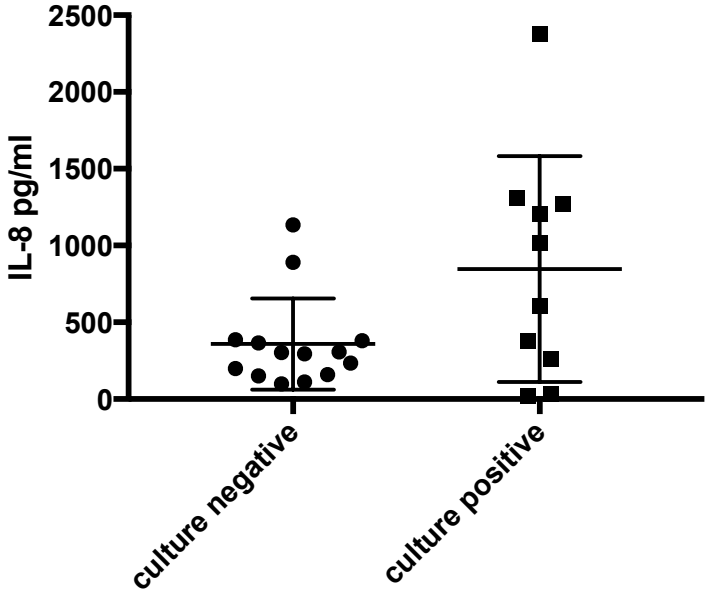


Figure 4.6 Baseline plasma IL-8 levels in the AAH patients who developed culture-positive infection and those who did not.

There was no significant difference between the 2 groups. Mann-Whitney test was used to analyse the data.

4.4.4 Neutrophil function – longitudinal analyses

There was no significant difference in day 1 phagocytosis compared to day 7 when the whole group was examined. Phagocytic capacity in the group who did not receive prednisolone showed a trend to improve at day 7 compared to day 1 (p=0.06) (Figure 4.7). An improvement was not seen in the prednisolone-treated group of patients (Figure 4.8). Day 7: day 1 fold change in phagocytic capacity was significantly higher in the group of patients not exposed to prednisolone compared to the group treated with prednisolone (p=0.01) (Figure 4.9). Eight patients had neutrophil function studies performed at day 30, in addition to day 1 and day 7. Three of these patients were in the prednisolone-arm, 5 were not treated with prednisolone. Figures 4.10 and 4.11 demonstrate the trends seen in these individuals.

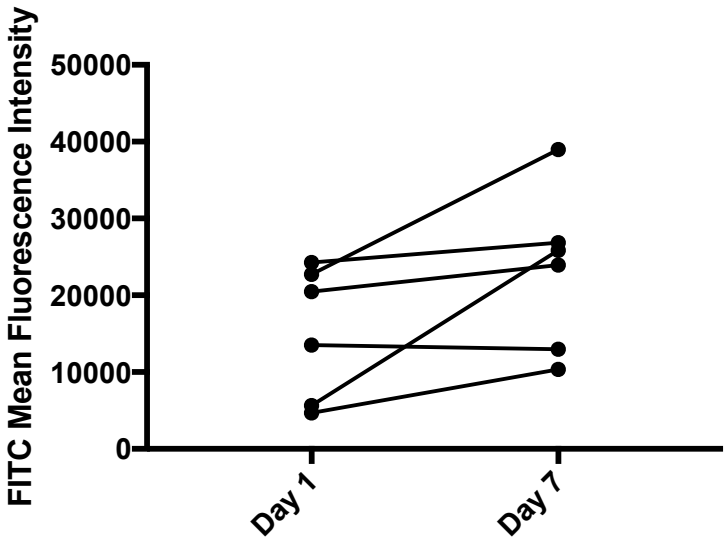


Figure 4.7 Phagocytic capacity in the AAH patients not exposed to prednisolone on day 1 and day 7.

A trend to improvement in phagocytosis on day 7 compared to day 1 was observed (p=0.06). Paired t-test was used to analyse the data (normal data).

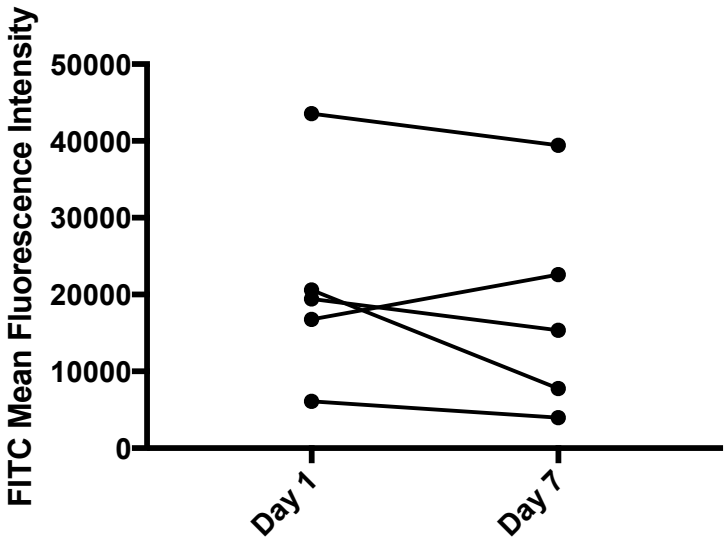


Figure 4.8 Phagocytic capacity in the AAH patients treated with prednisolone on day 1 and day 7.

No significant difference in phagocytosis on day 7 compared to day 1 was seen. Paired t-test was used to analyse the data (normal data).

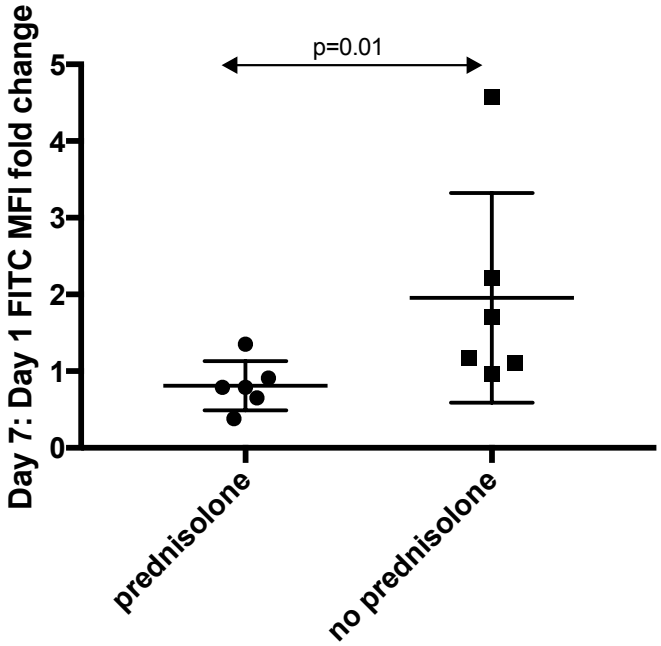


Figure 4.9 Day 7: day 1 fold change in phagocytic capacity in the prednisolone-treated AAH patients compared to the non-prednisolone exposed patients.

The fold change was significantly higher in the patients not exposed to prednisolone compared to the group treated with prednisolone (p=0.01). Mann-Whitney test was used to analyse the data.

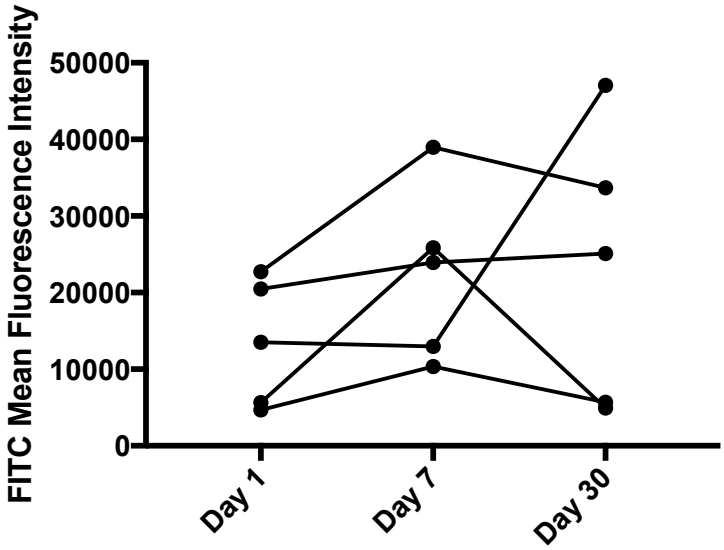


Figure 4.10 Phagocytic capacity in the AAH patients not exposed to prednisolone on day 1, day 7 and day 30, each line represents individual patient data.

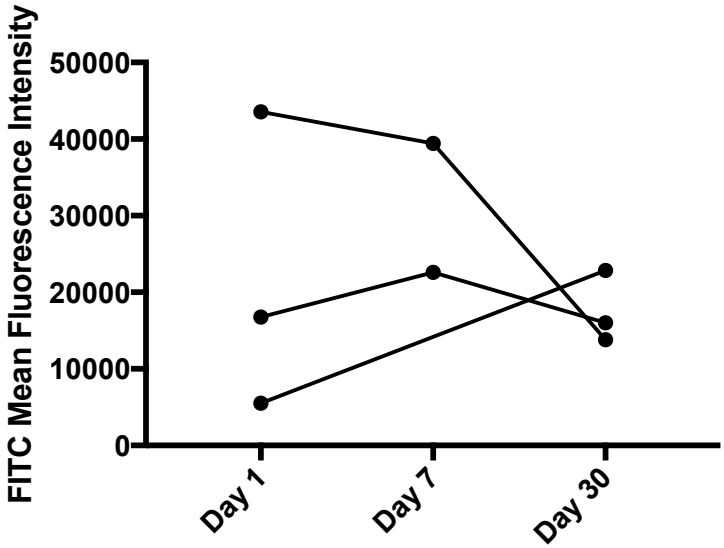


Figure 4.11 Phagocytic capacity in the AAH patients treated with prednisolone on day 1, day 7 and day 30, each line represents individual patient data.

The patient with the improved MFI at day 30 was alive at 6 months, no infection documented. The patient with the markedly reduced MFI at day 30 compared to day 1 died at 5 months.

There was no difference in resting burst between day 1 and day 7 in either the prednisolone-treated or non-prednisolone treated groups. When day 7: day 1 fold change in resting burst was compared between the two groups no significant difference was observed. Figure 4.12 demonstrates the changes observed in the small number of patients who had sequential analyses – day 1, day 7 and day 30. These patients were not enrolled into the prednisolone arm/treated with prednisolone.

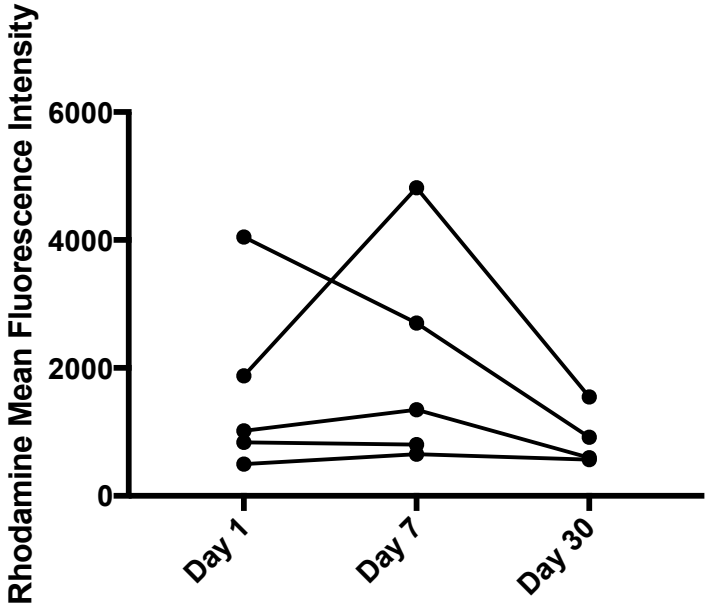


Figure 4.12 Resting burst in the AAH patients not exposed to prednisolone on day 1, day 7 and day 30, each line represents individual patient data.

4.4.5 Plasma IL-8, IL-6, lactoferrin – longitudinal analyses

Plasma IL-8 at day 7 was significantly lower than day 1 when the whole AAH group were examined (Figure 4.13). On sub-group analyses, IL-8 significantly fell in the group that were not treated with prednisolone (Figure 4.14). The difference between day 1 and day 7 levels was not significant in the prednisolone-treated patients. On removal of the outlier, the only patient found to have an increase in IL-8 at day 7, there was a trend of IL-8 reduction at day 7 compared to day 1 in the prednisolone-treated patients ($p=0.06$) (Figure 4.15). The day 1 plasma IL-8 levels tended to be higher in the group treated with prednisolone than the group who were not exposed to prednisolone ($p=0.06$). There was no significant difference in the fold change in IL-8 levels in day 1 and day 7 between the two groups.

Figure 4.16 demonstrates the changes observed in the small number of patients who had sequential analyses – day 1, day 7 and day 30. These patients were not enrolled into the prednisolone arm/treated with prednisolone. (Prednisolone-treated patients not shown as only 2 had day 1, day 7, day 30 IL-8 analyses).

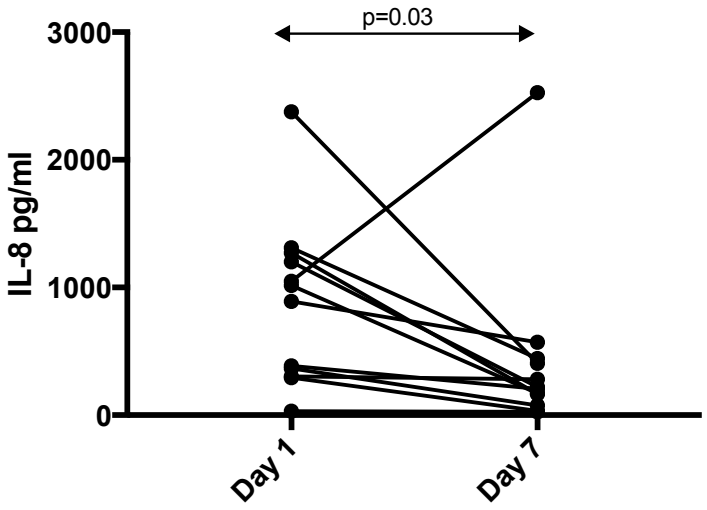


Figure 4.13 Plasma IL-8 levels in the AAH patients on day 1 and day 7.

IL-8 was significantly lower across the whole group on day 7 compared to day 1 (p=0.03). The outlier was included in analyses. Wilcoxon matched-pairs sign rank test was used to analyse the data.

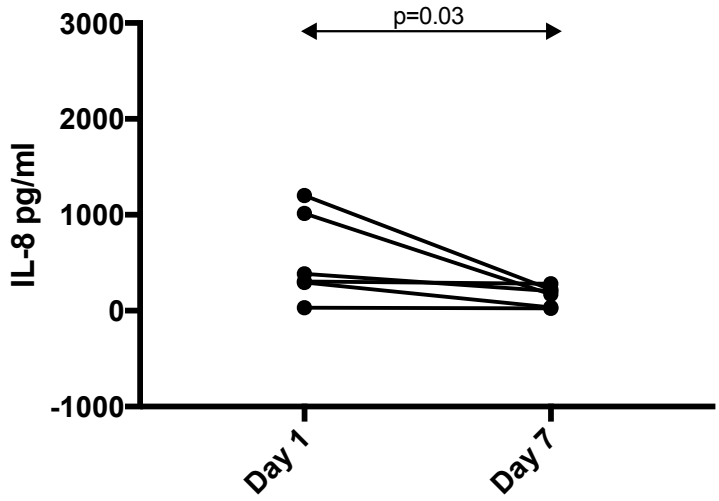


Figure 4.14 Plasma IL-8 levels in the AAH patients not exposed to prednisolone on day 1 and day 7.

IL-8 was significantly lower in this group on day 7 compared to day 1 (p=0.03). Wilcoxon matched-pairs sign rank test was used to analyse the data.

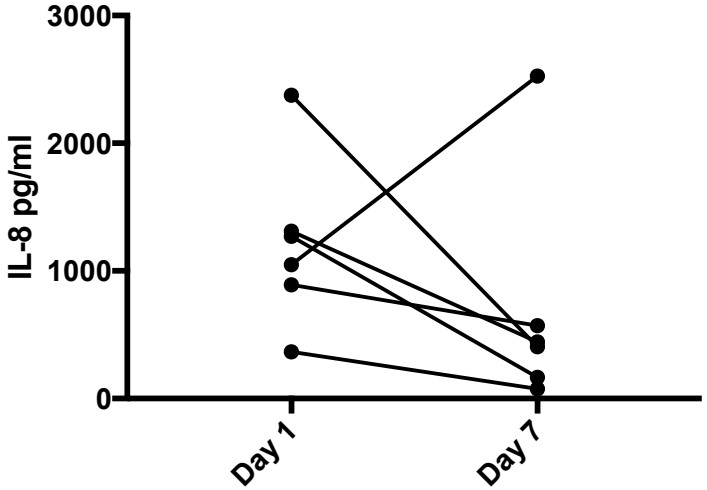


Figure 4.15 Plasma IL-8 levels in the AAH patients exposed to prednisolone on day 1 and day 7.

There was no significant difference in IL-8 on day 7 compared to day 1. When the outlier is removed there is a trend to reduction in IL-8 on day 7 compared to day 1 (p=0.06). Wilcoxon matched-pairs sign rank test was used to analyse the data.

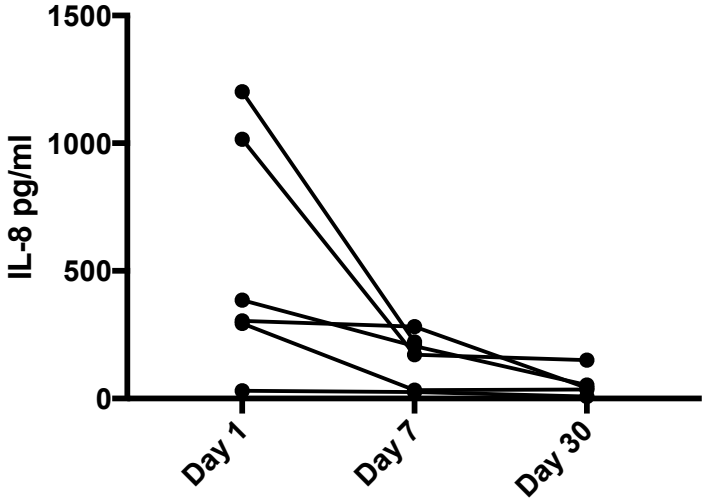


Figure 4.16 Plasma IL-8 levels on day 1, day 7 and day 30 in the AAH patients, these patients were not in the prednisolone arm/treated with prednisolone.

IL-6 at day 7 was significantly lower than day 1 when the whole AAH group were examined (Figure 4.17). There was a trend in reduction in IL-6 levels at day 7 compared to day 1 in the group treated with prednisolone (Figure 4.18). There was no significant difference in IL-6 levels at day 1 and day 7 in the group not exposed to prednisolone (Figure 4.19). There was no significant difference in the day 1 IL-6 levels between the group treated with prednisolone and the group not exposed to prednisolone.

Figure 4.20 demonstrates the changes observed in the small number of patients who had sequential analyses – day 1, day 7 and day 30. These patients were not enrolled into the prednisolone arm/treated with prednisolone.

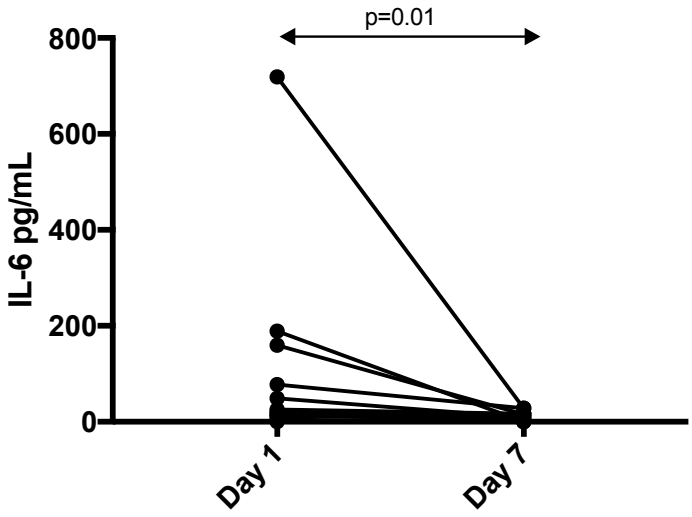


Figure 4.17 Plasma IL-6 levels in the AAH patients on day 1 and day 7.

IL-6 was significantly lower across the whole group on day 7 compared to day 1 (p=0.01).
This remained the case when the outlier (with a very high plasma day 1 IL-6 level) was removed (p=0.026).
Wilcoxon matched-pairs sign rank test was used to analyse the data.

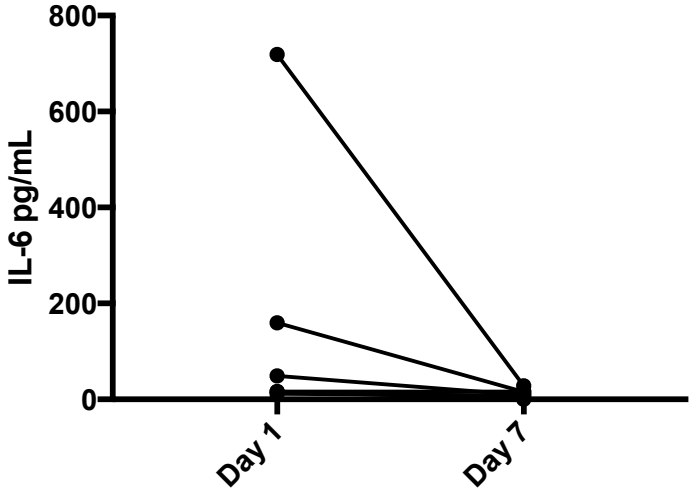


Figure 4.18 Plasma IL-6 levels in the AAH patients exposed to prednisolone on day 1 and day 7.

IL-6 tended to be lower in this group on day 7 compared to day 1 (p=0.06). Wilcoxon matched-pairs sign rank test was used to analyse the data. (p=0.1 if the outlier was removed from analyses).

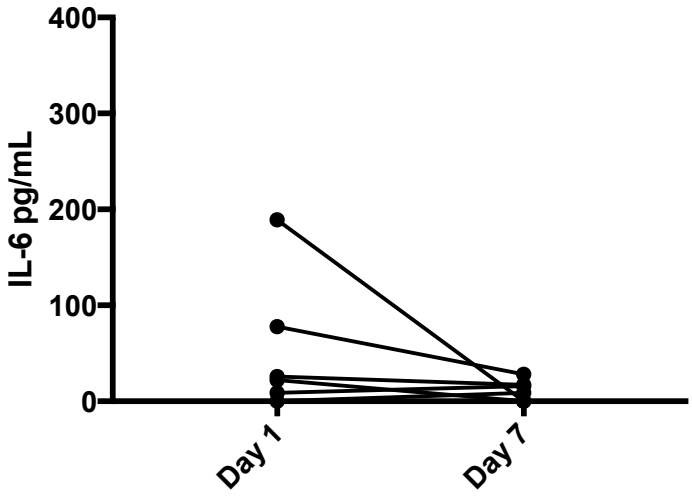


Figure 4.19 Plasma IL-6 levels in the AAH patients not exposed to prednisolone on day 1 and day 7.

There was no significant difference in IL-6 on day 7 compared to day 1. Wilcoxon matched-pairs sign rank test was used to analyse the data.

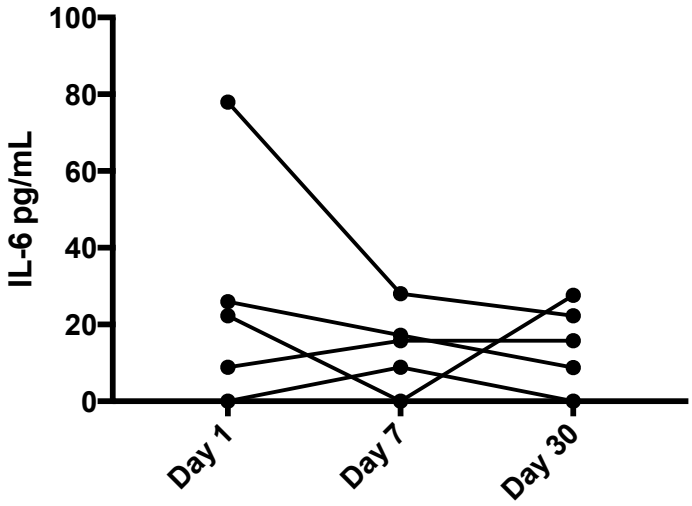


Figure 4.20 IL-6 levels at day 1, day 7 and day 30 in the AAH patients, these patients were not in the prednisolone arm/treated with prednisolone.

Although there was a trend to reduction in plasma lactoferrin at day 7 compared to day 1 no significant difference was seen when the whole group was examined and there was no difference seen on sub-group analyses.

4.5 Discussion

One-month mortality rates were perhaps lower than expected, this may have been impacted by the patients lost to follow-up, but is in line with results of the STOPAH study [98]. It was not possible to perform sequential analyses on all patients for similar reasons. In the small number of patients studied infection rates were not insignificant, patients were not recruited if there was evidence of untreated sepsis. The rate of infection may have also been under-represented as I chose to define infection as a positive culture over a time frame of 6 months post-sampling; this removed clinical bias and provided a robust definition for the follow-up period. The data could also have been analysed with definition of infection as positive urinalysis, chest X-ray or decision to initiate antibiotics; when the patients were divided in this way in my cohort the numbers were too small to run meaningful statistical analyses. The positive cultures were usually detected during the hospital admission or within 3 months of patient sampling.

Longer follow-up with sequential analyses to determine the most susceptible time point for infection would be of interest, as would correlation with response to steroids and liver recovery/regeneration. A recent publication using the STOPAH cohort suggests that infections may be predicted in AAH by measuring levels of circulating bacterial DNA [97]. The authors state that infections are common in AAH but are only independently associated with mortality when

patients receive prednisolone, and suggest that infection and mortality could be avoided by bacterial DNA stratified prednisolone-prescribing. The bacterial DNA data would be useful to study relative to the neutrophil analyses performed, and in particular in relation to the reduction in neutrophil function seen in the prednisolone group.

The numbers I studied were too small to provide clear-cut outcome correlations, but it appears that the neutrophil-TLR4 fold change post-LPS stimulation was significantly lower amongst the 6-month non-survivors compared to the 6-month survivors. Endotoxin-tolerance is now well recognized in cirrhotic patients [111] and these data point towards this as a poor prognostic feature in AAH. More than 90% of the AAH patients analysed in Vergis' recent study had detectable bacterial DNA levels [97]. As above, it would be interesting to correlate neutrophil function and TLR responses with bacterial DNA levels.

Taylor *et al* have previously shown that neutrophil phagocytic capacity and resting burst differentiates survivors from non-survivors in cirrhosis of all cause [152], the number of patients in this study was higher than the number of AAH patients included in my analyses which may, in part, explain the difference in findings. Of course, return to alcohol consumption is a key determinant when it comes to 6-month and longer-term survival in AAH and this would need to be kept in consideration with further study design. Of the 25 patients within my cohort who were not confirmed to have died at 3 months, 5 had definitely returned to alcohol use. This information was unknown in 10 patients who may have additionally returned to alcohol consumption by this early time-point.

Clearly, in further studies, when examining outcome after 3 months, patients would need to be stratified for return to alcohol use.

Perhaps the most notable finding is the impact of prednisolone on phagocytic capacity. Phagocytosis in the group who did not receive prednisolone showed a trend to improve at day 7 compared to day 1, conversely mean phagocytosis in the prednisolone-treated patients was seen to fall at day 7 and when the day 7-day 1 fold change were compared between the prednisolone-exposed and non-exposed groups a significant difference was observed. When this is taken into account along with the fact that STOPAH [98] showed serious infections occurred in 13% of patients treated with prednisolone versus 7% of those who did not receive prednisolone, this perhaps reinforces the concern that prednisolone therapy may be increasing the risk of infection in some individuals. Furthermore, prednisolone did not appear to temper the inflammatory response as measured by neutrophil ROS production, plasma lactoferrin or IL-8. There was, however, a trend to a reduction in plasma IL-6 in the prednisolone-treated group. Much of the literature on the effect of prednisolone on neutrophils comes from the respiratory and rheumatology fields. One study, however, has previously evaluated the effect of corticosteroids on some neutrophil markers, function and plasma IL-8 levels in 15 patients with AAH; Taieb *et al* observed that stimulated neutrophil CD11b and CD62L expression and H202 production returned to the levels seen in ARC and HC after 28 days of steroid therapy [61]. In addition the authors noted that during steroid therapy, plasma IL-8 levels were seen to fall as early as day 14. This study did not include a group of AAH patients unexposed to steroid treatment as controls and although, small

numbers, my data would suggest that the decrease in plasma cytokine levels seen may be part of the natural disease course and not influenced by steroid therapy. Neutrophil phagocytosis was not examined in Taieb's study. I did not sequentially investigate PBMC profiles or the IFN gamma/IL-10 skew, it is likely that the balance of cytokines in an individual is of key importance with regard to outcome and avoidance of infection and large comprehensive studies are required for further interrogation of this. In addition, five patients I studied were recruited from other centres and the treatment received was not known to me at the time of data analyses, re-analyses with this information may strengthen results.

Neutrophil adherence has, many years ago, been found to be reduced by corticosteroids, whereas no effect was seen on chemotaxis [212]. In severe steroid dependent asthma, prednisolone had no effect on neutrophil markers or on myeloperoxidase and neutrophil elastase release from purified neutrophils [213]. Steroids are known to inhibit apoptosis and increase survival in many cell types [214]. During my study, I began to develop apoptosis experiments but at a late stage and therefore failed to analyse any meaningful numbers of patients, this however, would be an additional aspect that might ultimately add to the understanding of the pathophysiology of AAH. The work would have been greatly enhanced if neutrophil lifespan studies had been concurrently conducted.

Unfortunately, the numbers of patients studied were too small to allow further sub-group analyses of innate immune function in steroid-responders compared to non-responders and this, of course, would be of particular interest.

The longer time-points of follow-up can only point towards trends given the numbers studied, but these highlight the individual dynamic course which, much like clinical outcome, is difficult to predict at the outset of illness. The day of presentation to hospital and previous presentations are also of importance.

In conclusion, this study provides further evidence of the potential negative immunosuppressive effects of prednisolone and the heterogeneity in individual trajectory within this disease which may well be influenced by past pathogen exposure and endotoxin tolerance.

These clinical findings thus led me to ask further questions, including why does ALD induce neutrophil dysfunction and do other derangements in neutrophil function contribute to the state of immunoparesis in ALD and these form the basis of chapters 5 and 6.

5 Use of the HL-60 cell line to examine mechanisms of neutrophil dysfunction and the relationship between neutrophils, ethanol and the liver

5.1 Background

Primary neutrophils are difficult to use in culture as they have a short life-span and are easily activated. Under certain conditions the HL-60 cell line differentiates toward a neutrophil-like cell. As described in chapter 1, the HL-60 cells have, therefore, been used to examine neutrophil biology and certain inflammatory conditions, but not previously in ALD.

My clinical observations posed further questions including why does ALD induce neutrophil dysfunction and do other derangements in neutrophil function contribute to the state of immunoparesis in this condition?

The HL-60 cells were thus used in this part of the study to further examine the relationship between neutrophils, ethanol and the liver. Through this work, further questions regarding neutrophil biology were encountered, shedding light on previously unknown aspects of neutrophil interactions both in health and ALD, discussed in chapter 6.

5.2 Aim of the investigation

The first aim of this study was to grow and differentiate the HL-60 cells and characterise phenotype and function of the differentiated and non-differentiated cells. The second aim was to examine the impact of ethanol and metabolised ethanol on neutrophil phenotype and function. Finally, given that type I and II interferon receptors, specifically, the IL-10 receptor, which is a subunit for IFN-λ, are present on neutrophils I questioned whether the differentiated cells express the IFN-λ receptor. At this point there was not a single published study describing IFN-λ receptor expression or the effect of IFN-λ on neutrophils.

Modulation of function was then examined which led to IFN-λ analysis on human neutrophils and the work detailed in chapter 6.

5.3 Methods

5.3.1 HL-60 cell culture

Cells were thawed and re-suspended in culture medium as described in 2.19. Cells were maintained initially in 20% FBS, then transferred to 10% FBS and maintained between 0.1-1 million cells/mL.

5.3.2 HL-60 differentiation and characterisation

Optimisation of differentiation was performed using different concentrations of DMSO (0.75%, 1.25% and 1.5%) and different time-frames of exposure. The expression of CD14, CD16, CD11b, TLR2, TLR4, TLR9, CD181 and CD182 on the differentiated and non-differentiated cells were examined by FACS as described in chapter 2. The Phagotest and Phagoburst experiments were adapted for use on the differentiated cells, described in the appendix.

5.3.3 HL-60 ethanol exposure

The phenotype and function of the differentiated HL-60 cells was assessed following culture with ethanol in concentrations of 10mM, 50mM, 100mM, 250mM and 500mM. In these experiments, hepatocyte (VL17A) metabolized ethanol was also used (courtesy of Dr Elena Palma Institute of Hepatology).

5.3.4 Interferon lambda stimulation

Phagocytosis of the differentiated HL-60 cells was examined following pre-treatment with recombinant IFN-λ1 (IL-29) (100ng/mL for 1 hour).

5.4 Results

5.4.1 HL-60 differentiation and characterisation

Table 5.1 outlines the HL-60 cell viability post thaw and pre-culture. Five passages were thawed for use, two were discarded due to contamination.

Table 5-1 HL-60 viability pre-culture

Date	Passage	Viability at thaw
01/10/2014	**3**	**78%**
10/10/2014	10	94% (contaminated and subsequently discarded)
28/10/2014	5	61% (no growth, discarded)
04/11/2014	**5**	**59%**
04/11/2014	**11**	**88%**

Figure 5.1 demonstrates the growth curve of the cell passages. Cells were divided regularly so that cell concentration did not exceed 1 million cells/mL.

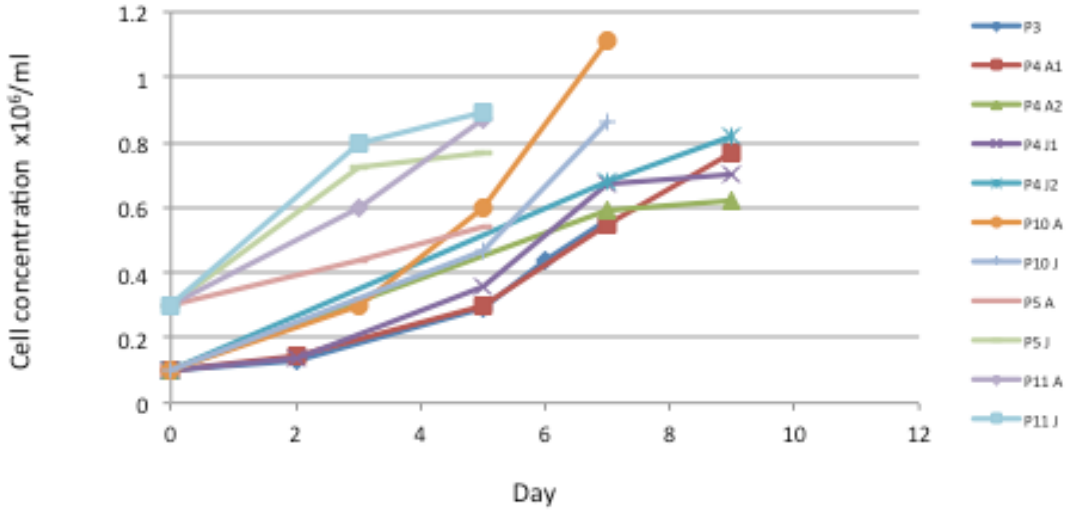


Figure 5.1 HL-60 growth curve. P delineates the passage of cells with cell concentration expressed as million per mL.

HL-60 cells were initially cultured with 1.5% DMSO for 7 days. Growth of cells exposed to DMSO was seen to reduce compared to those in culture medium alone (Figure 5.2). There were no dead cells seen in either condition when counted with trypan blue.

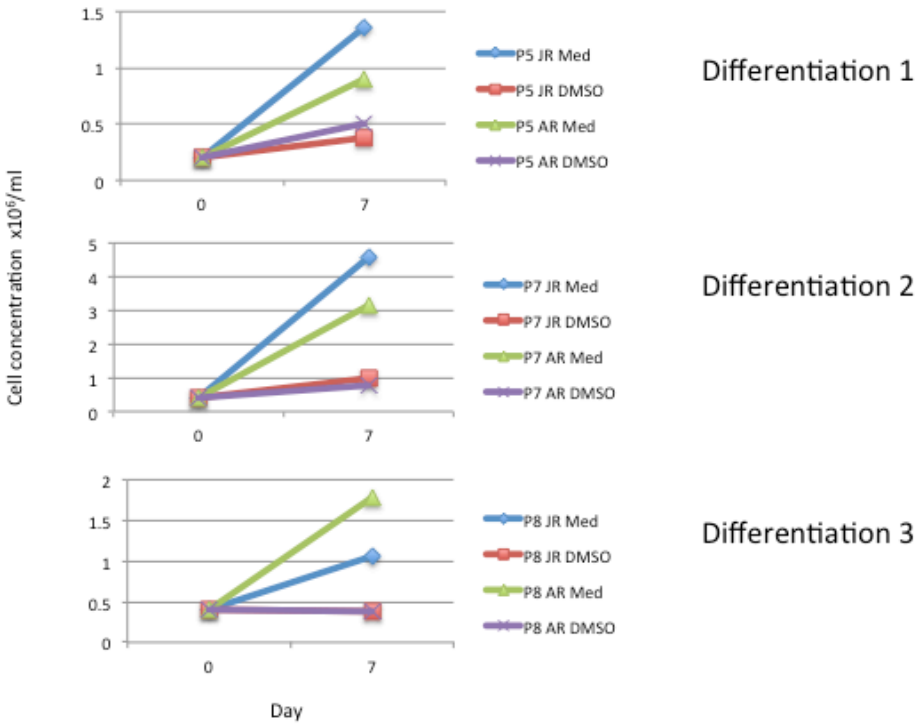


Figure 5.2 HL-60 cell differentiation with 1.5% DMSO. DMSO; dimethyl sulfoxide. Med; medium.

Morphological alterations of HL-60 during differentiation include shrinkage in cell size and decreased nuclear-cytoplasmic ratio (Figures 5.3 - 5.6).

Other features include increased nuclear segmentation and replacement of the coarse azurophilic granules with smaller specific granules.

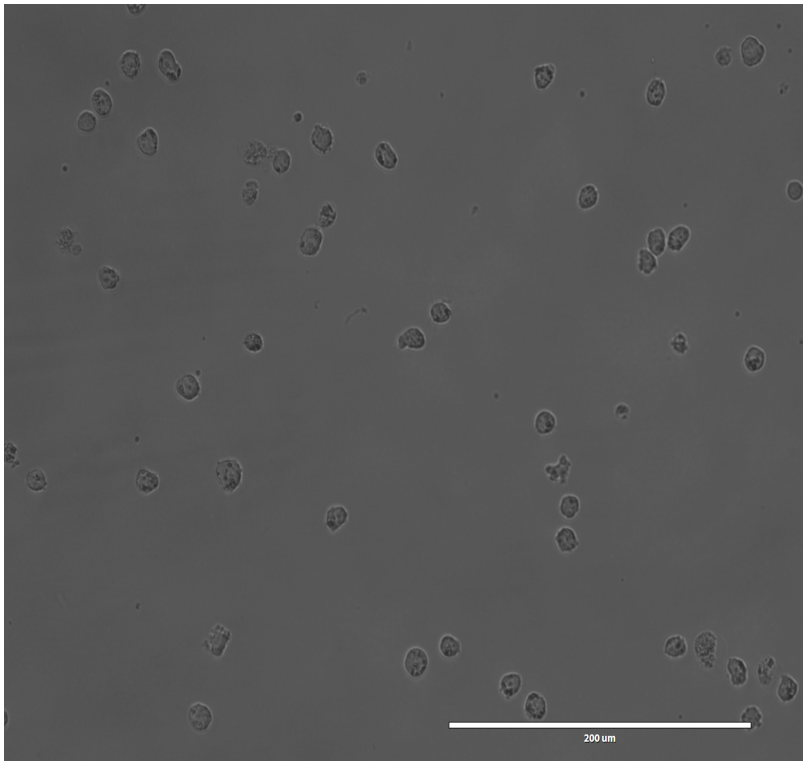


Figure 5.3 Microscopy photograph of HL-60 cells, passage 8 at day 1 in medium.

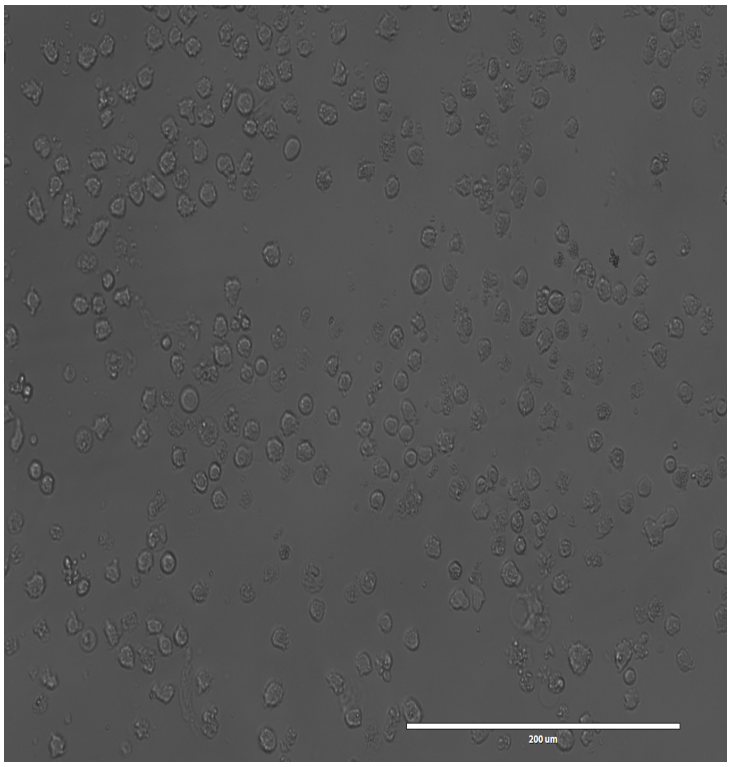


Figure 5.4 Microscopy photograph of HL-60 cells, passage 8 at day 1 in 1.5% DMSO.

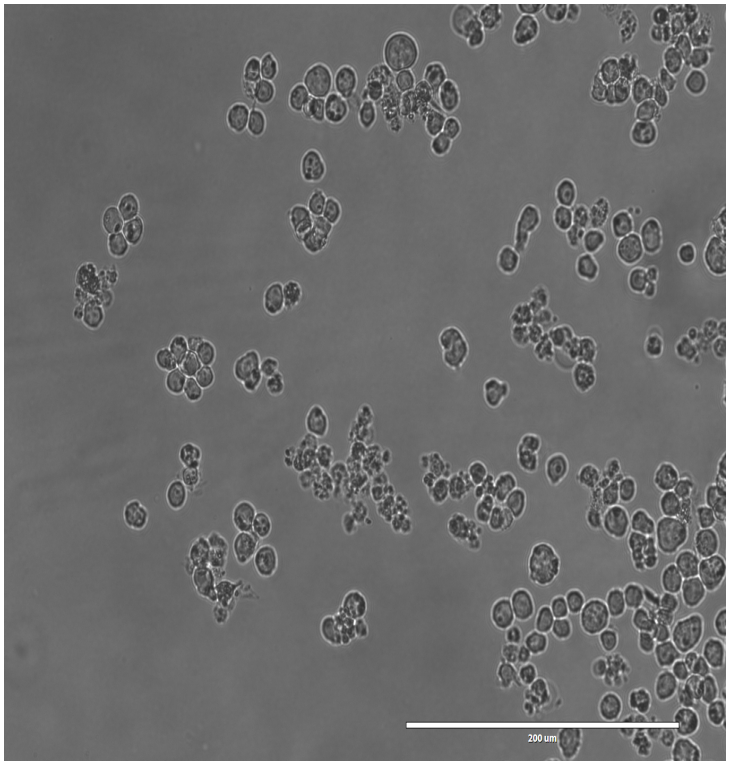


Figure 5.5 Microscopy photograph of HL-60 cells, passage 8 at day 7 in medium.

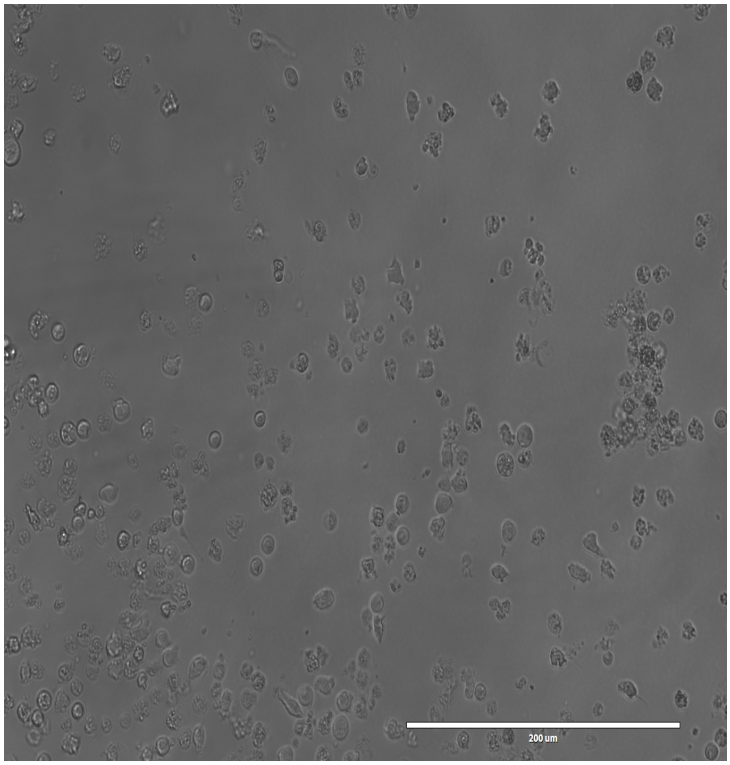


Figure 5.6 Microscopy photograph of HL-60 cells, passage 8 at day 7 in 1.5% DMSO.

After exposure to 1.5% DMSO for 7 days, in addition to the shrinkage of cells and slowed growth, the percentage of cells positive for CD11b, a marker of differentiation increased from 20% to 70% in all passages. 1.5% DMSO exposure also increased the proportion of CD16 positive cells. When the population was examined using the neutrophil gating strategy, the CD16 positive and CD11b positive percentage of cells increased upon 1.5% DMSO exposure (Figure 5.7). When several passages were exposed to increasing concentrations of DMSO (0.75%, 1.25%, 1.5%) the highest concentration of DMSO used resulted in the highest percentage of cells positive for CD16 and CD11b.

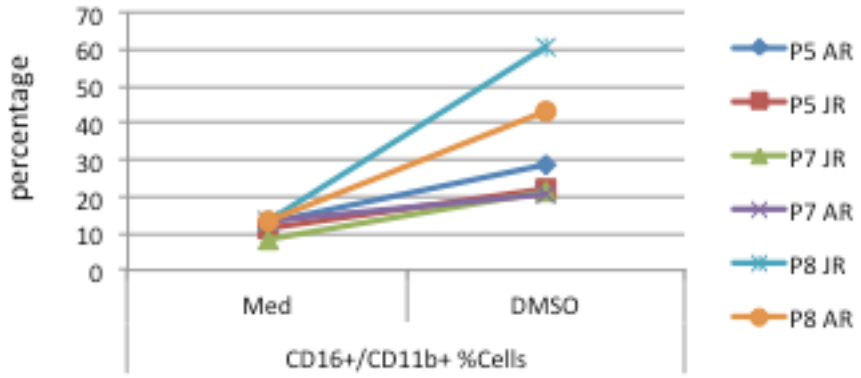


Figure 5.7 CD16 and CD11b positive percentage of cells.

An increase in proportion of cells positive for both markers was seen after exposure to DMSO compared to the cells kept in medium alone. The individual lines represent individual passages.

Cells were then exposed to 1.5% DMSO for 5, 6 or 7 days and phenotype examined. CD14 receptor expression was also induced by DMSO exposure, but, unlike CD16 and CD11b expression change, the fold change reduced with the number of days of exposure (Figure 5.8 and 5.9).

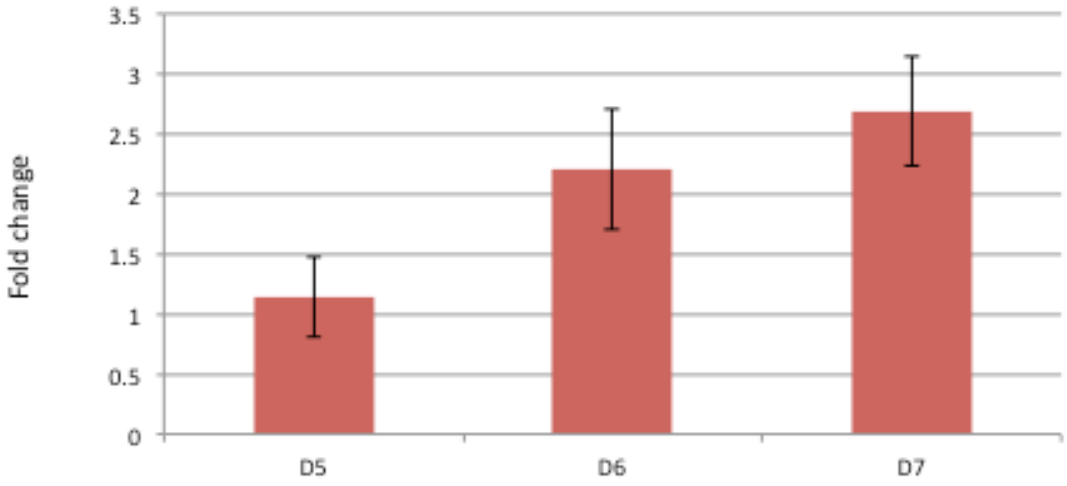


Figure 5.8 CD16 and CD11b positive population fold change in DMSO exposed cells compared to cells not treated with DMSO at day 5, day 6 and day 7.

An increase in fold change in the cells positive for both CD16 and CD11b was seen with time.

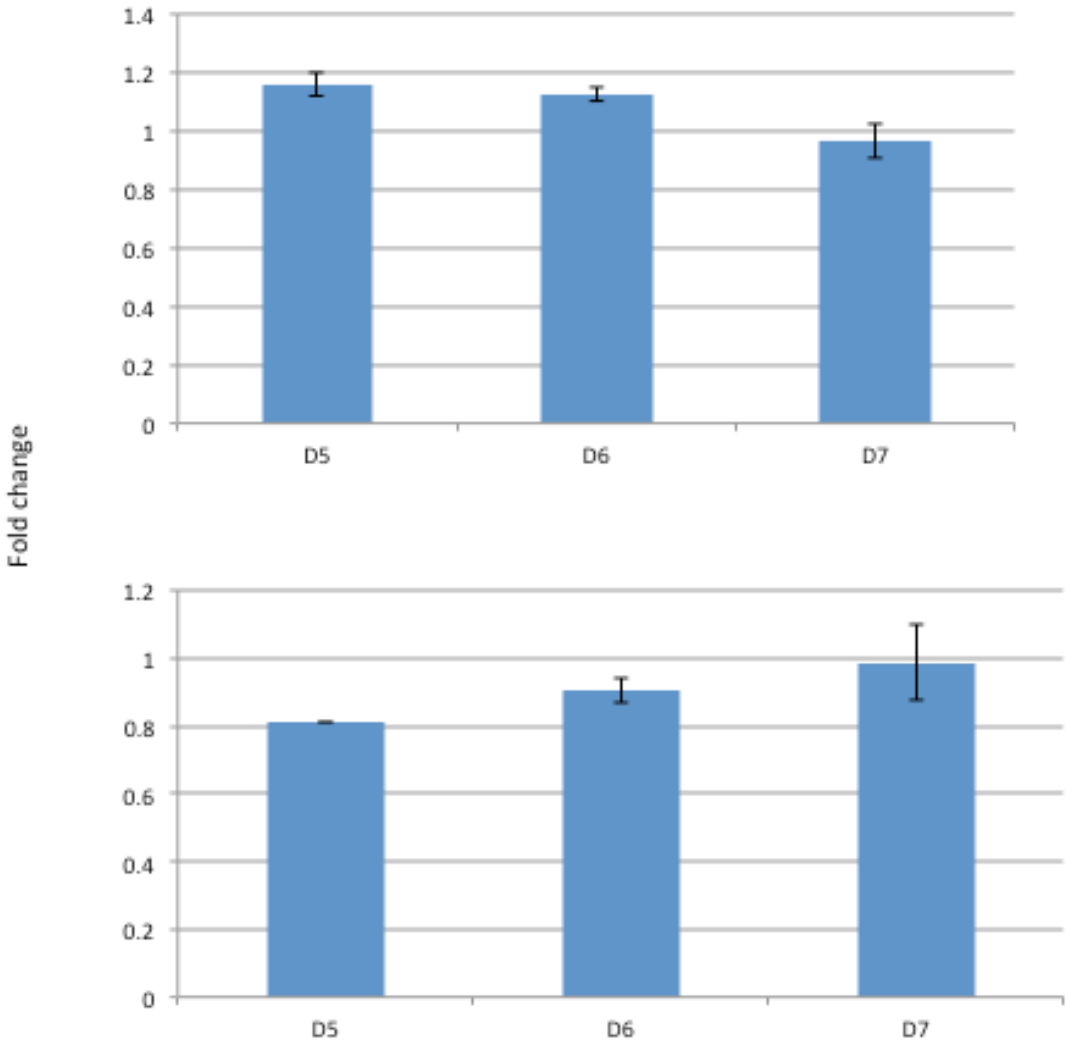


Figure 5.9 Fold change in surface expression markers on differentiated HL60.

The CD16 and CD11b positive cells were gated.
Fold change in the population positive for both CD14 and CD16 amongst this population on exposure to DMSO at day 5, day 6 and day 7 (top) was seen to reduce with time.
In contrast, fold change in the CD14 negative and CD16 positive population on exposure to DMSO at day 5, day 6 and day 7 (bottom) was seen to increase with time.

Differentiated and non-differentiated cells were found to express TLR2, 4 and 9 (Figures 5.10 – 5.12). The CD181 receptor was minimally expressed by the HL-60 cells and exposure to DMSO resulted in a variable response between passages. The CD182 receptor, in comparison to CD181, was expressed on a higher percentage of cells and DMSO induced expression (Figure 5.13).

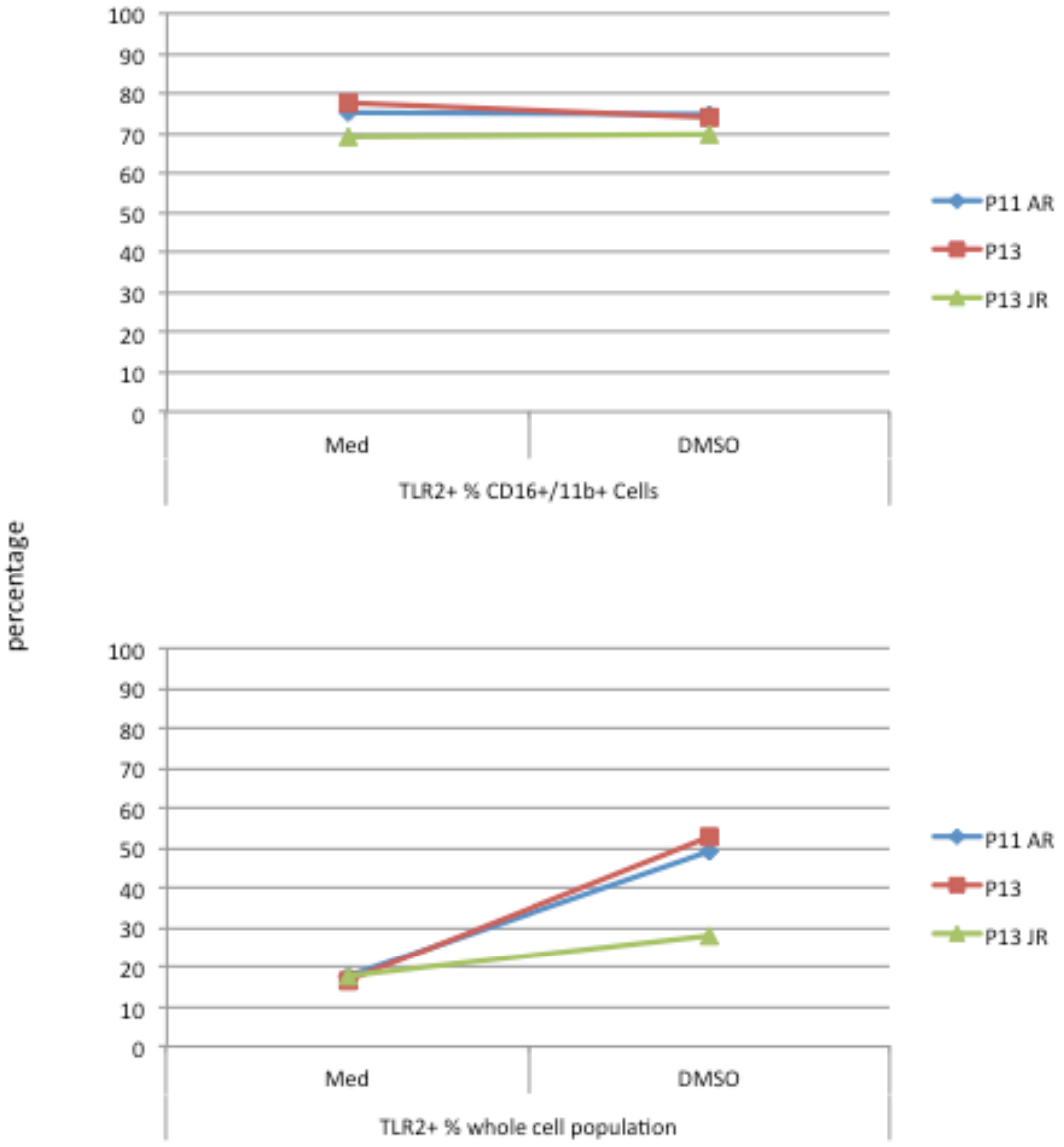


Figure 5.10 TLR2 expression on HL60 cells.

TLR2 positive percentage of CD16 and CD11b positive population (top), cells exposed to 1.5% DMSO for 6 days compared to cells kept in culture medium alone. TLR2 positive percentage of the whole population (bottom), cells exposed to 1.5% DMSO for 6 days compared to cells kept in culture medium alone.

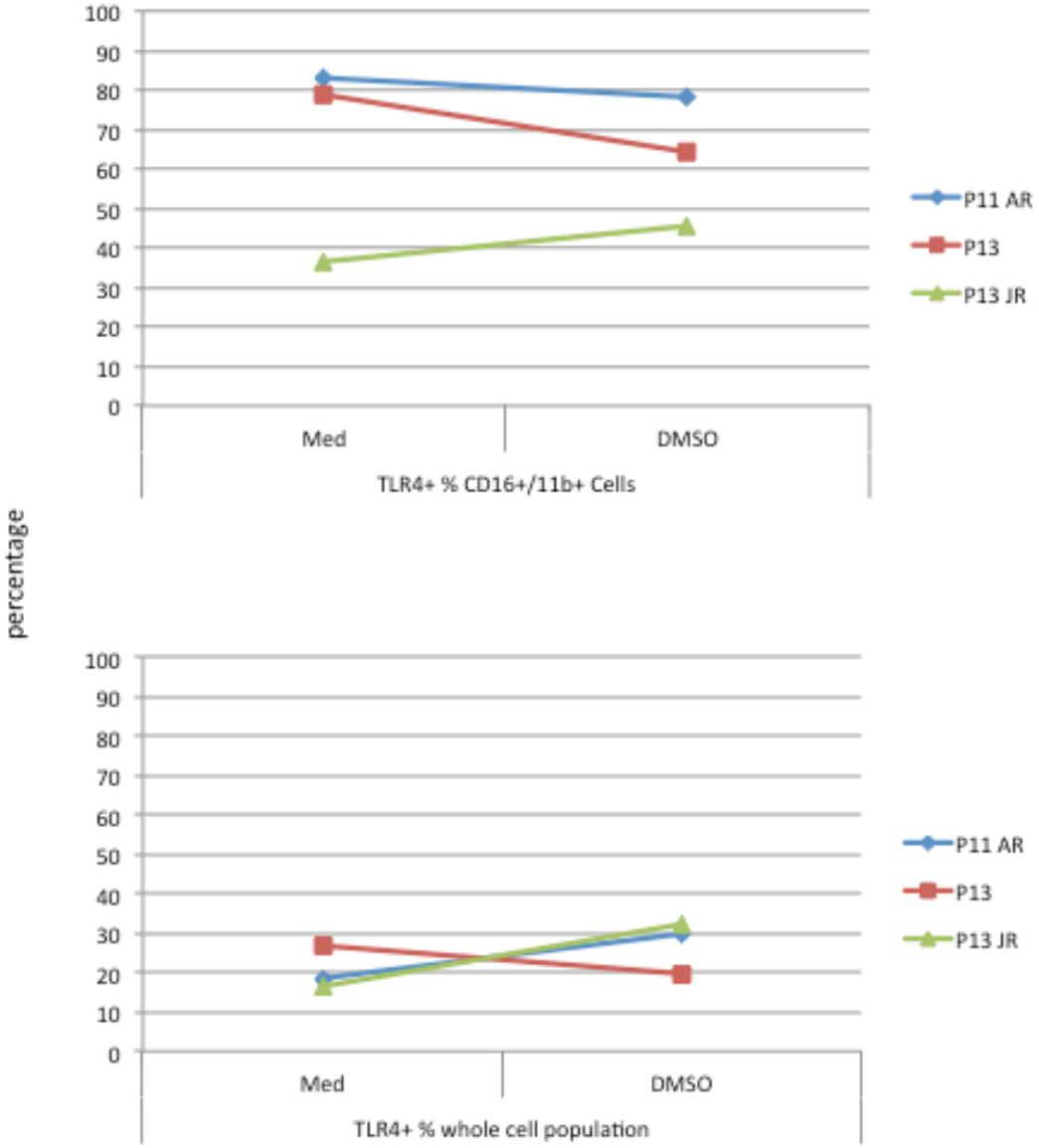


Figure 5.11 TLR4 expression on HL60 cells.

TLR4 positive percentage of CD16 and CD11b positive population (top), cells exposed to 1.5% DMSO for 6 days compared to cells kept in culture medium alone. TLR4 positive percentage of the whole population (bottom), cells exposed to 1.5% DMSO for 6 days compared to cells kept in culture medium alone.

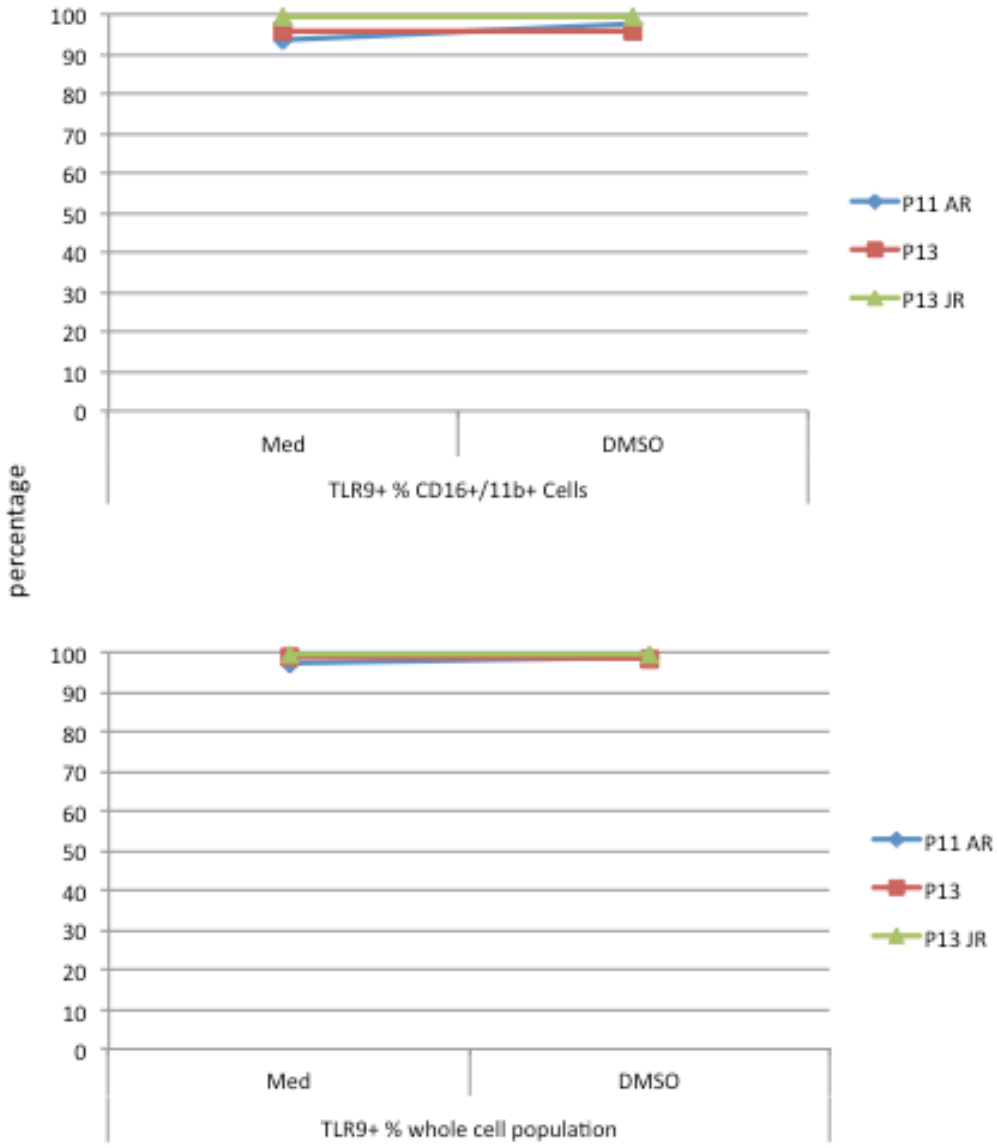


Figure 5.12 TLR9 expression on HL60 cells.

TLR9 positive percentage of CD16 and CD11b positive population (top), cells exposed to 1.5% DMSO for 6 days compared to cells kept in culture medium alone. TLR9 positive percentage of the whole population (bottom), cells exposed to 1.5% DMSO for 6 days compared to cells kept in culture medium alone.

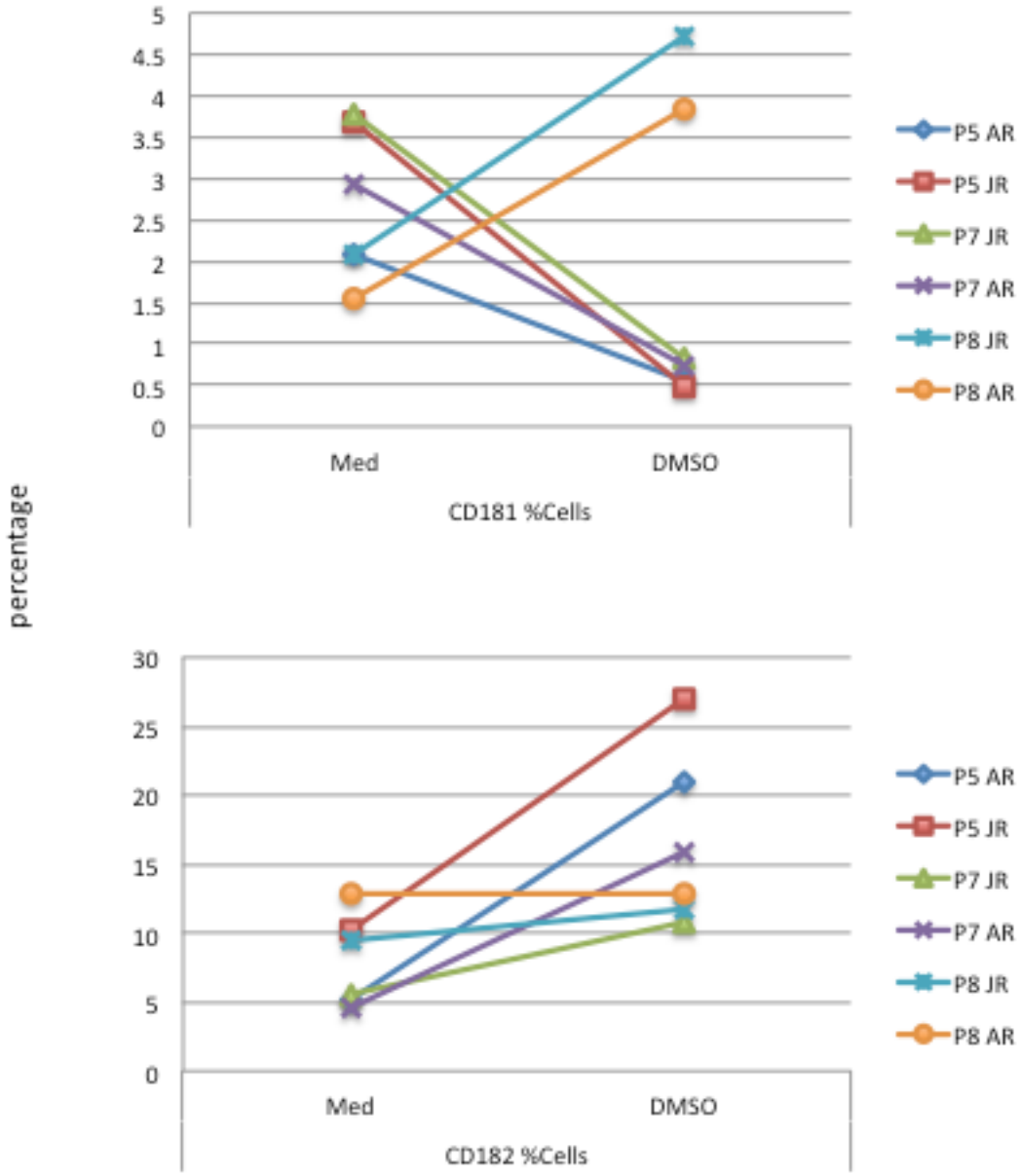


Figure 5.13 CD181 and CD182 expression on HL60 cells.

CD181 positive percentage of HL-60 cells (top), cells exposed to 1.5% DMSO for 6 days compared to cells kept in culture medium alone. CD182 positive percentage of cells (bottom graph), cells exposed to 1.5% DMSO for 6 days compared to cells kept in culture medium alone.

Using the Phagoburst kits, very high levels of resting burst were seen with both the differentiated and non-differentiated cells on several passages.

Phagocytic activity was examined following differentiation with 0.75, 1.25 and 1.5% DMSO as outlined in figure 5.14. Initially 200uL of labeled *E. coli* was cultured with 100, 000 cells for 4 hours. (A ratio of 2000 bacteria to cell was recommended by kit manufacturers). A low number of events were obtained when the cells positive for CD16 and 11b were analysed. The whole cell population was additionally studied in sequential experiments.

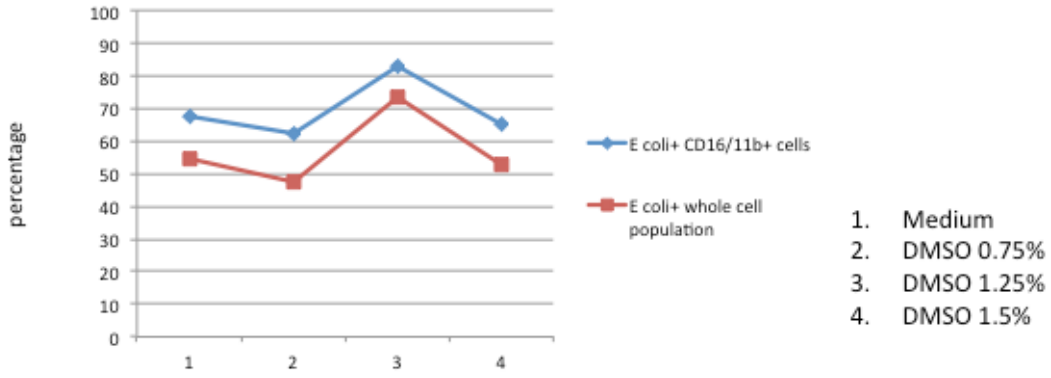


Figure 5.14 Phagocytic Activity of HL-60 cells,

Phagocytosis of HL-60 cells, non-differentiated (medium) and differentiated (cells cultured with 0.75, 1.25 and 1.5% DMSO for 6 days). The red line represents the whole population, the blue line represents the population of CD16 and 11b positive cells.

Next, experiments were performed using a titration of *E. coli* and phagocytic activity was seen with lower concentrations of *E coli* (Figure 5.15).

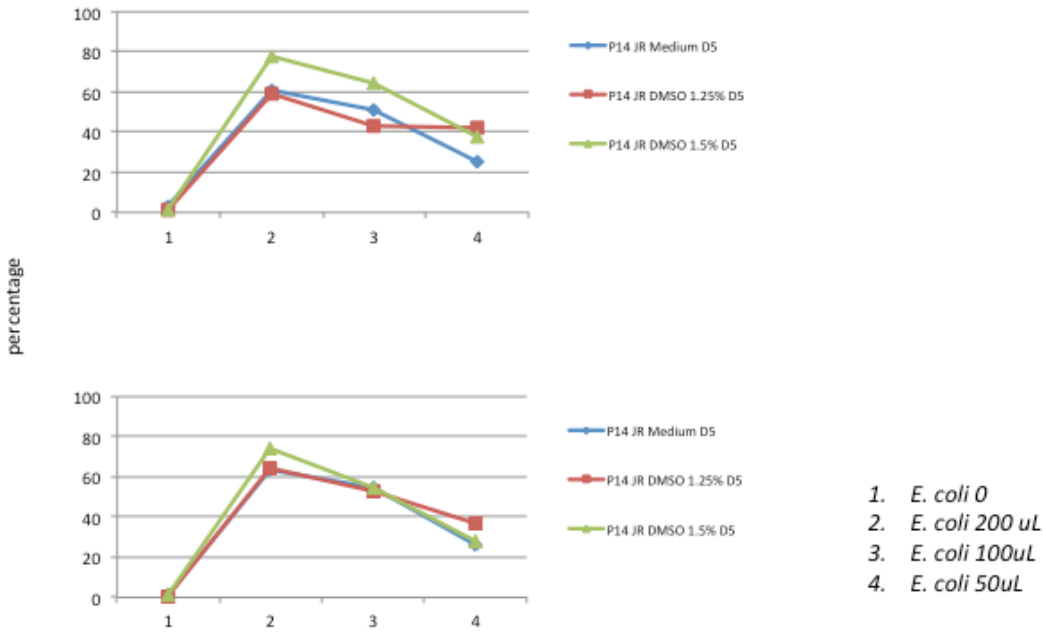


Figure 5.15 Titration of *E.coli* for HL-60 phagocytosis experiments.

The top graph represents the phagocytic activity of the CD16 and CD11b positive population of the non-differentiated cells (blue), the cells exposed to 1.25% DMSO (red) and the cells exposed to 1.5% DMSO (green). The bottom graph represent the phagocytic activity of the whole population of cells. Condition 1 – control, 2- 200uL *E.coli,* 3 - 100uL *E.coli, 4* - 50uL *E.coli.*

Based on both phenotype and function data, it was decided that conditions for differentiation for ongoing experiments would be exposure of the HL-60 to 1.5% DMSO for 6 days. Differentiated cells generally showed higher phagocytic activity than the non-differentiated cells; the latter were also seen to phagocytose however. Phagocytosis was also examined using opsonised and non-opsonised bacteria, on ice and at 37 degrees centigrade. The differentiated cells showed reduced phagocytosis with non-opsonised bacteria at 37 degrees (Figure 5.16). Cells were noted to phagocytose on ice and this was seen when lower volumes of *E. coli* were used. Cells were viewed with fluorescent microscopy following *E. coli* exposure. When cells were kept on ice the bacteria was predominantly seen to be adherent to the cell surface as opposed to the cells cultured at 37 degrees where the labeled bacteria were seen to be internalised (Figures 5.18 and 5.19).

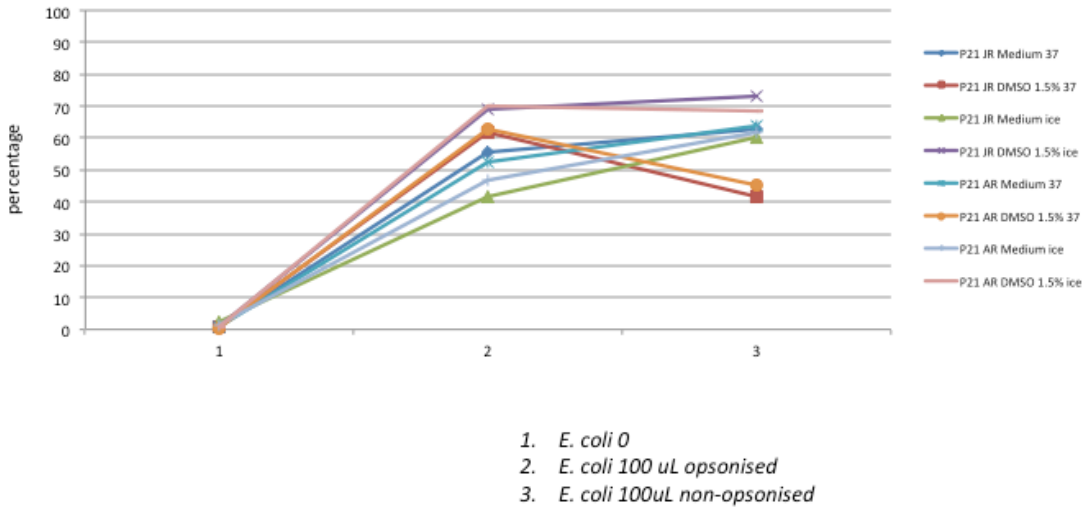


Figure 5.16 Phagocytosis of opsonised and non-opsonised *E.coli* on ice and at 37 degrees of the differentiated and non-differentiated cells.

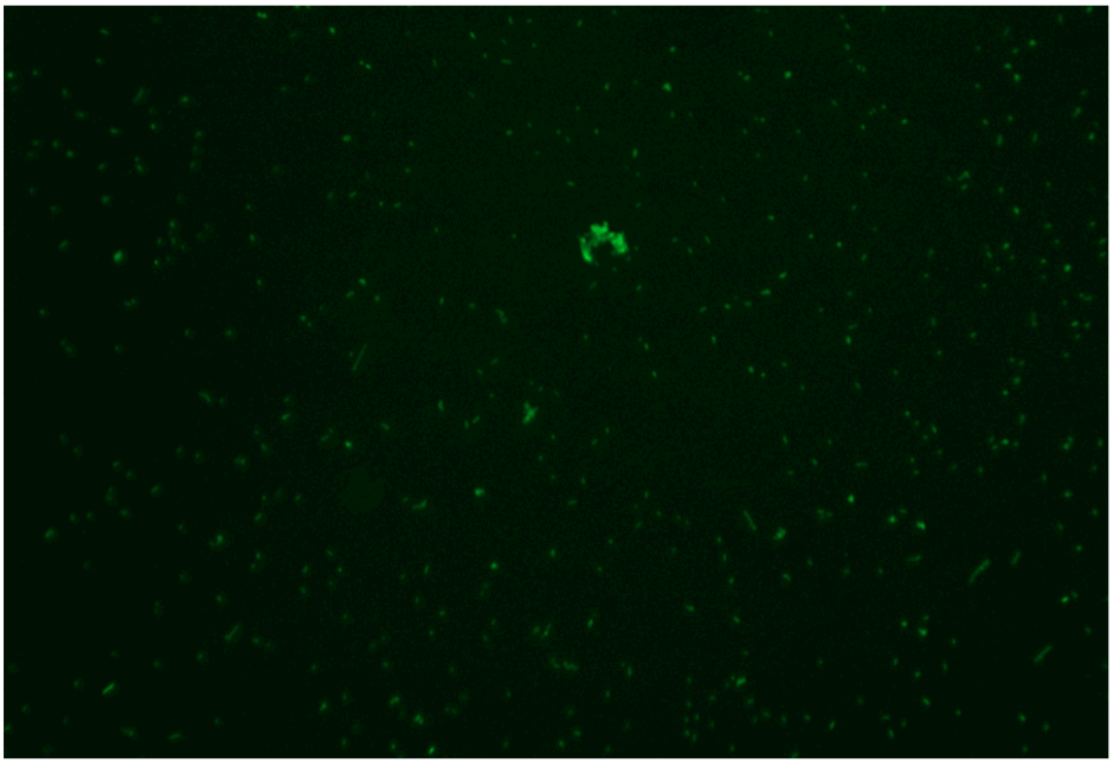


Figure 5.17 Microscopy of HL-60 cells and labelled *E. coli* on ice.

When cells were kept on ice bacteria were adherent to the cell surface.

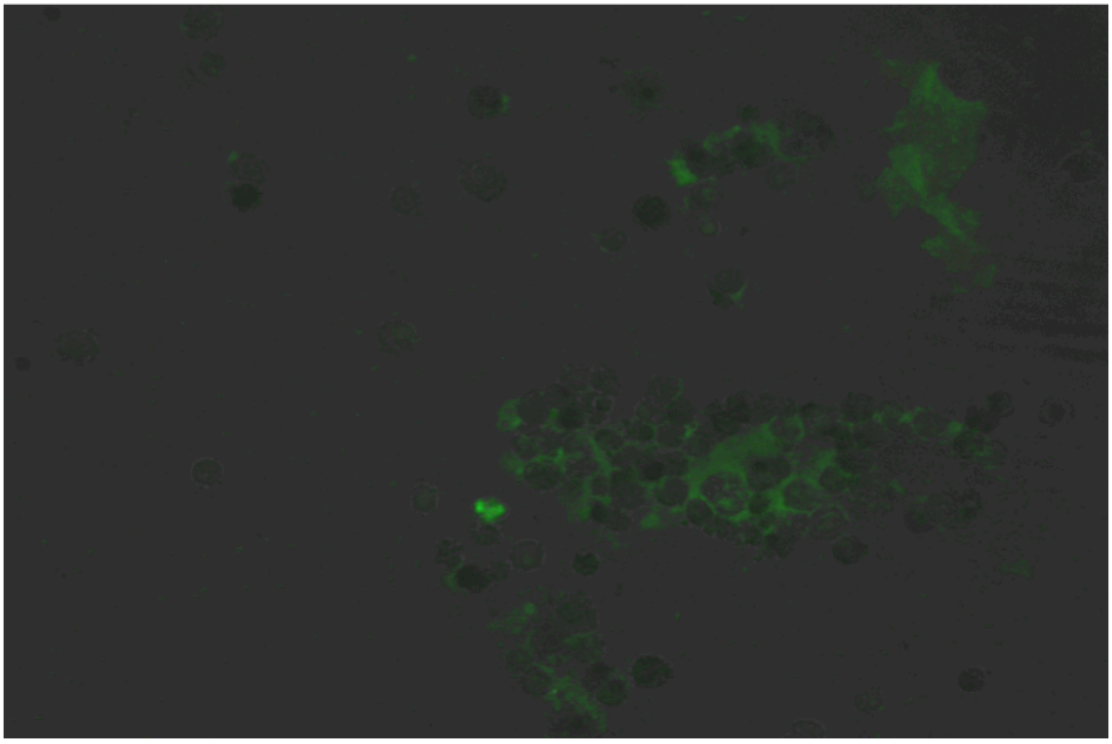


Figure 5.18 Microscopy of HL-60 cells and labelled *E. coli* incubated at 37 degrees.

Bacteria is seen within cells with evidence of NET formation.

5.4.2 Impact of ethanol on HL-60 cells

Culture of the differentiated HL-60 cells with ethanol and VL17A-metabolised ethanol resulted in reduced expression of CD16, CD11b, TLR2 and 4. There was no change seen in the expression of TLR9. Increasing concentrations of ethanol were found to reduce phagocytic activity of the cells (Figure 5.19). VL17A-metabolised ethanol reduced phagocytic activity of the differentiated HL-60 cells compared to differentiated HL-60 control cells and cells exposed to the supernatant of the non-ethanol treated VL17A cell line (Figure 5.20). VL17A-metabolised ethanol further reduced phagocytosis of the differentiated HL-60 cells compared to ethanol alone (Figure 5.20).

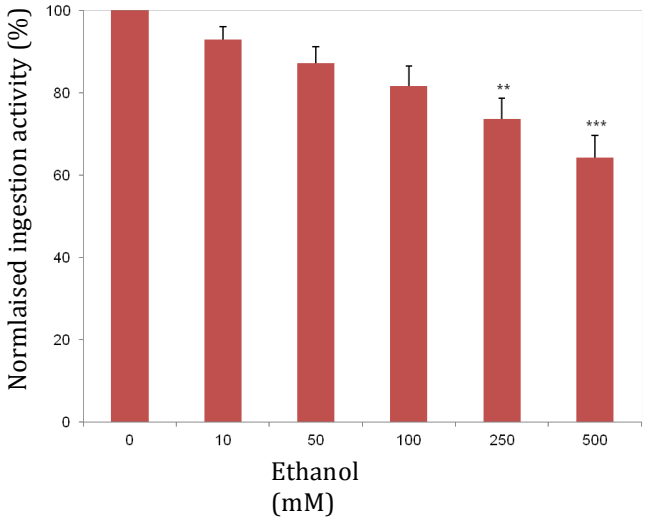


Figure 5.19 Differentiated HL-60 cell phagocytic capacity reduced with increasing concentration of ethanol exposure.

* - compared to control cells. ** - $p<0.01$, *** - $p<0.005$. One-way analysis of variance used.

Figure 5.20 Differentiated HL-60 phagocytic activity reduced on exposure to VL17A-metabolised ethanol.

There was no difference in phagocytic activity between control cells and cells cultured with supernatant from the VL17A line, not exposed to ethanol. Phagocytic activity of the differentiated HL-60 cells following exposure to ethanol and VL17A-metabolised ethanol.

5.4.3 Modulation of differentiated HL-60 function by interferon lambda

First, differentiated HL-60 cells were examined for the IL-10 receptor and IL28 receptor α chain (IFN-λr) using FACS. The cells expressed IL-10 but did not express the IFN-λr. Several passages were analysed and intracellular and extracellular staining for IFN-λr was performed. Following pre-treatment with recombinant IFN-λ1 phagocytosis increased by 10-20% (Figure 5.22). This was confirmed on the analyses of several passages of cells.

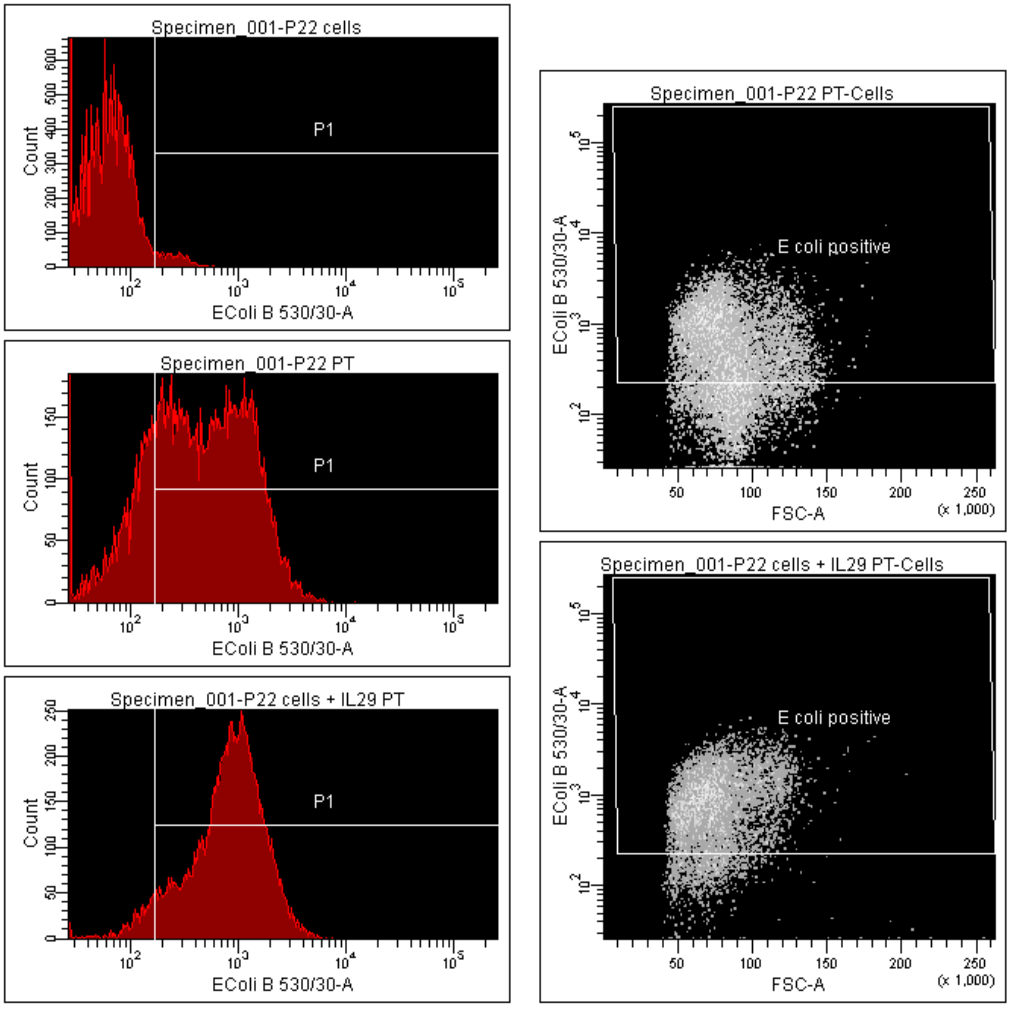


Figure 5.21 FACS plots of the differentiated HL-60 cells quantifying phagocytosis post treatment with IFN-λ1.

Top left – control differentiated HL-60 cells not cultured with labelled *E. coli.* Middle left and top right – differentiated HL-60 cells phagocytosing. Bottom left and bottom right – increased phagocytosis of the differentiated HL-60 cells following treatment with IFN-λ1.

5.5 Discussion

After optimisation it was possible to differentiate the HL-60 cells towards a neutrophil-phenotype. One of my early concerns was that a significant proportion of cells became CD14 positive. CD14 is a myeloid cell differentiation antigen expressed primarily by monocytes and macrophages. Studies in the early 1990s, however, showed that neutrophils also express CD14 and can respond to LPS through a similar pathway as monocytes [215]. I demonstrated that the fold change in CD14 gradually reduced as the number of days the cells were exposed to DMSO increased; this was in contrast to the CD16 and CD11b fold change. Exposure to 1.5% DMSO for 6 days was eventually chosen as the optimal differentiation conditions given the combination of phenotypic and function data. The time-frame was also influenced by the practical aspects of planning and completing my experiments. Various conditions for neutrophil differentiation have been described [160], the use of DMSO and all-*trans*-retinoic acid (ATRA) being the most common. ATRA, however, does not induce the expression of CD16, CD66 and CD88 antigens, normally expressed on granulocytes [216] on HL60 cells. I therefore did not go on to use this agent for differentiation.

I demonstrated that these cells expressed many of the key receptors examined in the human study. In particular, these cells may act as a good model for further study of TLR9 as this was highly expressed pre- and post-differentiation. The expression of TLRs, however, does not appear to be a marker of differentiation of these cells, at least toward a neutrophil-like state. A previous study found TLR4 expression to be only slightly induced in ATRA-treated HL-60 cells (HL-60-

derived granulocytic cells), and strongly induced in IFN-γ-treated HL-60 cells (HL-60-derived monocytic cells) [217].

Both HL-60 cells and differentiated HL-60 were found to phagocytose, however, the differentiated cells did show higher phagocytic activity. It was possible to reduce the ratio of bacteria to cell recommended by the kit manufacturers. The labeled *E. coli* supplied in the phagotest kits is opsonised with immunoglobulin and complement of pooled sera. When phagocytosis was examined in the most physiological-like conditions – using opsonised *E. coli* at 37 degrees – phagocytosis of the differentiated HL-60 cells of greater than 60% was observed. Perhaps, unsurprisingly, there was a marked decrease in this when non-opsonised bacteria was used at 37 degrees. This acts as further evidence that these cells have been differentiated toward a granulocyte-like state. Phagocytosis was observed when cells were kept on ice and it was postulated that bacteria were adherent to the cell surface rather than internalised in these conditions. This was indeed confirmed on fluorescent microscopy of cells. This is a drawback when using the flow cytometric opsonophagocytosis assays and it is, of course, recognised that these assays measure binding and/or phagocytosis and not necessarily killing of bacteria [160].

Despite modifications of the conditions, high levels of baseline oxidative burst were observed both in the medium and differentiated cells. It was therefore not meaningful to examine burst induced by *E.coli* or other stimulants, and this was one of the limitations of this work. The labeling and quantitative analysis of myeloperoxidase or the examination of culture-supernatants for pro-inflammatory cytokines could potentially be used as a surrogate for examining

resting burst and the influence of certain agents on these cells. This would, however, need further exploration.

There appeared to be a direct effect of ethanol on the cell surface markers, with resultant reduced expression. There have been limited studies investigating the direct impact of ethanol on neutrophil phenotype and function. This is perhaps reflective of the difficulty of excluding other factors in such analyses. One study performed in critically ill non-trauma patients compared C-reactive protein (CRP) values, circulating subsets of white blood cells, and neutrophil CD64 indexes recorded at admission to the intensive care unit between abstinent or moderate drinkers, patients with acute on chronic alcohol exposure, and patients with acute exposure but not chronically exposed to alcohol. Values for CRP, circulating neutrophils, and neutrophil CD64 indexes were significantly lower in patients acutely exposed compared with the other patients [218]. The authors also found increased numbers of circulating B and T lymphocytes in the patients with acute exposure to alcohol and concluded that acute alcohol may diminish inflammation in these patients. Despite the recognition of the impact of ethanol on innate and adaptive immunity in patients with cirrhosis there have been few studies that have attempted to look at the direct impact of ethanol.

No change was seen on intracellular TLR9 following ethanol exposure. Although much more work would be needed to investigate the increased TLR9 I detected in my AAH patients, the lack of induction by ethanol on these cells potentially points towards other factors, such as DAMPs, as discussed in chapter 3, being the predominant driver for the increase seen and these cells could be used further to explore this. For example, the cells could be examined after exposure to

stimulants thought to be involved in sterile inflammation. One of the benefits of using the cells was that extended culture with ethanol was possible.

Ethanol appeared to have a negative impact on phagocytosis and, interestingly, the VL17A-metabolised ethanol decreased phagocytosis further. From the limited experiments performed it is difficult to postulate what it is within the hepatocyte-metabolised ethanol that could be potentiating the negative effect and this would be interesting to look at. Most studies investigating the impact of ethanol on neutrophil function have looked at short-term exposure. Animal studies have reported mixed effects of ethanol on neutrophil function [219, 220]. When the influence of acute ethanol on phagocytic function of neutrophils against Klebsiella Pneumoniae in humans was investigated, the phagocytic function, as well as other neutrophil functions, such as adhesion, chemotaxis, and oxygen metabolism, were suppressed with acute ethanol intoxication [221]. Of course, mimicking the environment of the milieu of the circulating AAH neutrophils is, *in vitro*, difficult, as is mimicking the environment of chronic ethanol use but these cells could be used in further experimental models to allow the investigation of specific aspects of neutrophil biology in this context. In addition they could be used in co-culture with the VL17A cells (Figure 5.23), whereby the impact of certain stimulants and therapies could be further examined by assessing neutrophil function and hepatocyte death in both direct and indirect cultures.

I did not examine the differentiated HL-60 cells post LPS-exposure and given my previous findings and the likely importance of endotoxin tolerance in ALD this

was a significant limitation of this part of the work. This, in itself, is another research project and would need to include the exploration of whether these cells provided a viable model for 7-day and extended periods of culture. The cells have previously been evaluated for their suitability as an *in vitro* model system to study the responses of neutrophils to TLR2/4 ligand exposure, the incubation period used was chosen as 4 hours [222]. Given the evolving importance of the microbiome and different 'favourable' and 'non-favourable' strains of bacteria this could also be examined within this model. In addition, the cells could also be used to further explore neutrophil-responses to fungal pathogens clearly of importance in ALD [223].

There are, of course, limitations encountered with the use of the HL-60 cells. Despite careful handling of the cells, there was variability seen between the passages. They represent a cancer-cell line and despite differentiation of the cells this would need to be kept in mind specifically when evaluating certain therapies. With reference to some of my previous findings in the initial human study, the HL-60 cells lack lactoferrin mRNA. This could potentially be used to an advantage, however, in terms of assessing the contribution of lactoferrin to microbicidal activity or inflammation for example.

Despite the limitations of these cells, in my work their study enabled an exploration of basic neutrophil biology. The exploration of the IFN-λ receptor and exogenous IFN-λ effect led to further questions regarding the relationship between neutrophils and IFN-λ which is discussed in chapter 6.

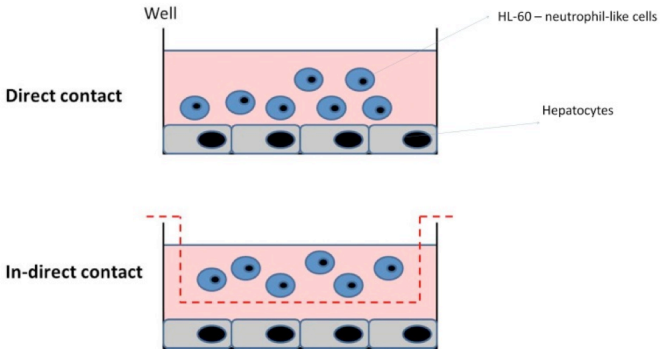


Figure 5.22 Potential co-culture model: neutrophil cell line and / or isolated neutrophils from some ALD patients are co-cultured with hepatoma cell lines in direct and indirect culture.

6 The neutrophil-interferon lambda relationship in alcohol-related liver disease

6.1 Background

IFN-λ has a restricted cell-response pattern and is thought to be of specific importance in antiviral protection at epithelial surfaces. Very little is known about the role of IFN-λ in bacterial infections and the relationship between this class of cytokine and neutrophil function. Recently, Blazek *et al.* have demonstrated that IL-28A targets neutrophil chemotaxis and impacts upon pathology in a mouse model of inflammatory arthritis [183]. The interplay between IFN-λ and neutrophils in other diseases has not been explored and the question as to whether neutrophils produce IFN-λ both in healthy and disease states remains unanswered.

6.2 Aim of the investigation

The first aim was to examine previously collected plasma samples from patients with ALD for IFN-λ1.

Next, healthy control blood was cultured with various stimulants to determine whether neutrophil IFN-λr was present and whether an IFN-λ response was, indeed, induced. Following results from the above, the contribution of neutrophil-specific IFN-λ in anti-bacterial immune defenses in patients with ARC was examined as described.

6.3 Methods

6.3.1 High sensitivity IFN-λ1 ELISA

A high sensitivity ELISA for IFN-λ was performed as per protocol on the plasma of HC n=5, AAH patients n=5, ARC patients n=10 and patients with hepatitis C virus (HCV) cirrhosis n=5.

6.3.2 Healthy control IFN-λ induction experiments

Whole blood cultures on HC n=5 were set up in parallel, with the following stimulants for 2 hours:

1. Gram positive bacteria – Pansorbin
2. Gram negative bacteria – *E. coli* - 10 bacteria per cell
3. Poly I:C
4. Control – PBS

One set of the above samples underwent neutrophil extraction using the MACSxpress Neutrophil Isolation Kit (2.9) providing a neutrophil fraction for RNA extraction. RNA was extracted directly from the other 4 samples (the leukocyte fraction). RNA was extracted from the neutrophil and leukocyte fraction as outlined in 2.20.5. Quantitative PCR was carried out using specific primers and probes for IFN-λ1, IFN-λ2/3, IFN-λr and RPLP0 as endogenous control as described in 2.20.6. RNA was also extracted from the differentiated HL-60 cells to include in the IFN-λr PCR.

6.3.3 IFN-λ examination post *E. coli* culture in ALD

Patients with ARC, n=11, were recruited and compared with HC, n=8. Whole blood was incubated with fixed *E. coli* at a concentration of 10 bacteria/cell or PBS (unstimulated) at 37°C for 2 hours as described in 2.21.7. Two parallel cultures were set up and after 2 hours, neutrophils were isolated immediately as described previously for RNA extraction and the whole blood fractions (both stimulated and unstimulated) were stored in RNAprotect Cell Reagent (Qiagen, UK) at -80°C for subsequent RNA extraction. RNA extraction and PCR were performed as described above. To understand whether the addition of

exogenous IFN-λ1, -λ2 or -λ3 could alter neutrophil function we cultured whole blood with recombinant cytokines and assessed neutrophil cell surface markers, phagocytosis and oxidative burst before and after cytokine treatment as described in 2.20.7. Plasma and post-IFN-λ supernatants were collected by centrifugation and stored at -80°C for subsequent cytokine quantification and high sensitivity IFN-λ1 ELISA as previously described.

6.4 Results

6.4.1 High sensitivity IFN-λ1 ELISA

IFN-λ1 was not detected in the plasma of the HC. It was, however, detected in 4 out of the 15 patients initially studied; 3 ARC and 1 AAH. The highest level was seen in a patient recovering from Streptococcus Gordonii SBP.

6.4.2 Healthy control IFN-λ induction

IFN-λr mRNA expression was demonstrated in the neutrophil population and also in the differentiated HL-60 cells. IFN-λ1 and IFN-λ2/3 mRNA was detected in the neutrophil fraction only after whole blood stimulation. *E. coli* was found to be the strongest stimulant compared to Poly I:C and the Gram-positive stimulant used.

6.4.3 Neutrophil-specific production of IFN-λ1 is deficient in advanced ARC

Baseline clinical characteristics of the patients studied are outlined in table 6.1. None of the patients had clinical infection at the time of sampling.

Table 6-1 Baseline clinical characteristics of the patients examined for neutrophil-specific IFN-λ response post *E. coli* challenge

Pt	Age	Sex	Bilirubin µmol/L	Albumin g/L	INR	Creatinine µmol/L	Ascites	HE	Child Pugh score	MELD	Group	Positive bacterial culture $	1-month outcome	6-month outcome
1	61	M	90	39	2	222	yes	no	C10	29	ABA	yes	alive	deceased
2	38	F	91	38	1.5	43	yes	Gd 3	C10	17	AA	no	alive	alive
3 *	46	M	23	42	1.1	70	no	no	A5	8	AA	no	alive	alive
4	56	F	15	28	1.3	28	yes	Gd 1	B9	9	AA	no	alive	alive
5 #	43	F	372	25	2.6	53	yes	Gd 2	C14	29	AAH	yes	alive	alive
6	45	M	13	23	1.5	274	yes	no	B9	22	ABA	no	alive	transplanted
7	56	M	16	29	1.2	217	yes	no	B8	17	AA	no	alive	alive
8 #	45	M	651	24	2.2	77	yes	no	C12	29	AAH	yes	deceased	deceased
9	58	M	37	33	1.7	85	yes	Gd 1	C11	16	ABA	no	alive	alive
10	60	M	44	25	1.	58	no	Gd 3	C10	15	ABA	no	alive	alive
11	68	M	19	38	3.8	212	yes	no	B9	30	AA	yes	alive	alive

* Patient admitted with variceal bleed
# Patients with alcoholic hepatitis on background of cirrhosis
$ Patients were followed-up for 6 months, those who became culture positive did so within 1-month of sampling
No patients were found to have a positive fungal culture within the study period

Expression of mRNA encoding IFN-λ1 was detected only after stimulation with *E. coli* in purified neutrophils and the total leukocyte fraction in both HCs and patients (Figure 6.1a). In contrast, *E. coli* challenged IFN-λ2/3 was undetectable or minimally detectable within the neutrophil and leukocyte fractions in both groups of subjects (Figure 6.1c). IFN-λr was detected basally and also following stimulation in the neutrophil and leukocyte fractions (Figure 6.1d).

The most notable findings were seen in the neutrophil expression of IFN-λ1. Amongst the patients two groups emerged; those who did not have *E. coli*-stimulated induction of IFN-λ1 (Group 1) and those who did (Group 2) (Figure 6.1a, 6.1b), although at a significantly lower level than the HCs (Figure 6.2a). *E. coli* stimulated neutrophil expression of IFN-λ1 in Group 1 was significantly less than in controls (p=0.001) and Group 2 (p=0.002) (Figure 6.2a). On further group analyses it was observed that bacterially challenged production of IFN-λ1 in the leukocyte fraction was also compromised in Group 1 (p=0.029) (Figure 6.2c). Interestingly, Group 1 had significantly higher Child-Pugh score and AST compared to Group 2 (p=0.029 and p=0.015, respectively). Therefore patients

that failed to induce IFN-λ1 response to bacterial challenge had more advanced liver disease. The increased Child-Pugh stage may also reflect higher circulating endotoxin levels (Table 6.2). Neutrophil resting oxidative burst was significantly higher in Group 1 compared to Group 2 ($p=0.047$) and plasma IL-8 was significantly higher in Group 1 compared to Group 2 ($p=0.017$).

The profound difference observed in IFN-λ1 between the patient groups was not mirrored in the neutrophil or leucocyte expression of IFN-λ2/3 and IFN-λr expression (Figure 6.1c, 6.1d). However, within the patient cohort there was a more variable response in neutrophil IFN-λr expression compared to HCs (Figure 6.1d).

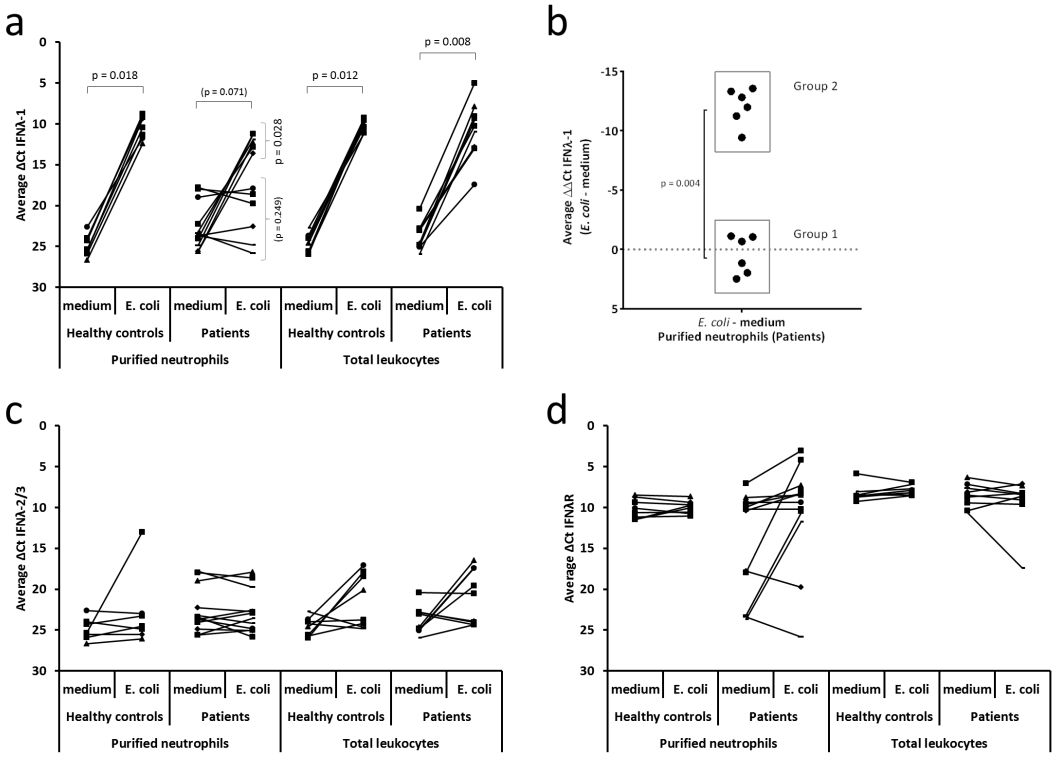


Figure 6.1 Deficient neutrophil mRNA expression of IFN-λ1 upon *E coli* stimulation.

(a) *E coli* induced production of IFN-λ1 mRNA in purified neutrophils and total leukocytes, expressed as ΔCT to control gene, in healthy controls and patients. Wilcoxon matched-pairs sign rank test was used to analyse the data. (b) Two groups are seen within the patient cohort when neutrophil IFN-λ1 mRNA is analysed, with markedly different response to *E coli.* Mann-Whitney test was used to analyse the data. (c) Minimal change was observed in neutrophil and total leukocyte IFN-λ2/3 mRNA following *E coli* in both healthy controls and patients. Wilcoxon matched-pairs sign rank test was used to analyse the data. (d) IFN-λr mRNA was detected in purified neutrophils and total leukocytes basally and after stimulation in both healthy controls and patients, there was no correlation between IFN-λ1 and IFN-λr mRNA induction. Wilcoxon matched-pairs sign rank test was used to analyse the data.

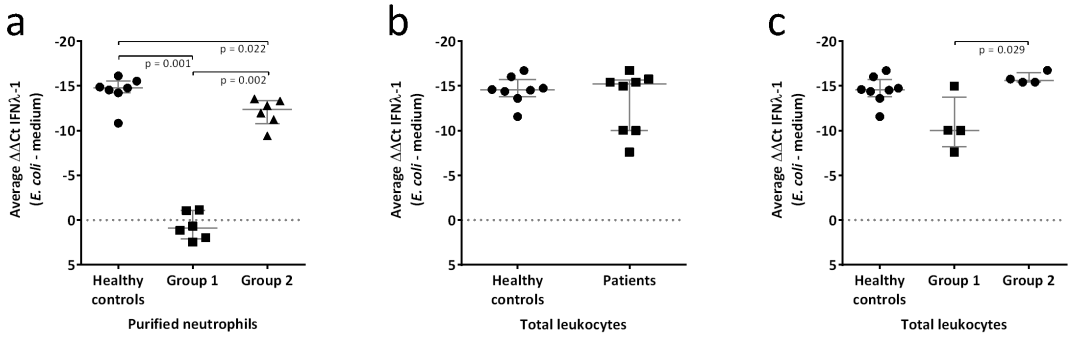


Figure 6.2 IFN-λ1mRNA expression, represented as ΔΔCT post- and pre- stimulation with *E. coli*.

(a) Patients were seen to divide into two distinct Groups; Group 1 who did not show stimulated induction of IFN-λ1 mRNA and Group 2 who did demonstrate induction, although at a lower level than the healthy controls. Kruskal-Wallis test with Dunn's multiple comparisons was used to analyse the data. (b) There was no difference in the change in leukocyte IFN-λ1 mRNA post stimulation between healthy controls and patients. However, on further analyses, Group 1 did show reduced induction whilst Group 2 showed similar induction to healthy controls Kruskal-Wallis test with Dunn's multiple comparisons was used to analyse the data. (c). Grey lines indicate the median value; whiskers indicate the interquartile range.

Table 6-2 Clinical and neutrophil function data for group 1 and group 2 patients.

Group 1 – patients who lacked interferon-λ1 induction upon *E. coli* stimulation, Group 2 – patients who had *E. coli*-stimulated induction of IFN-λ1.

Parameter	Group 1 vs Group 2 (p-value)	Group 1 (median - [IQR])	Group 2 (median - [IQR])
Age y	0.074	45.0 - [40.5, 52.5]	56.0 - [46.0, 61.0]
Bilirubin µmol/L	**0.015**	372.0 - [67.5, 719.5]	19.0 - [15.0, 37.0]
Albumin g/L	0.163	25.0 - [24.5, 31.5]	33.0 - [28.0, 39.0]
Child Pugh score	**0.029**	12.0 - [10.0, 14.5]	9.0 - [8.0, 10.3]
AST IU/L	**0.015**	126.0 - [70.8, 167.0]	50.0 - [33.0, 58.0]
Neut. CD182 post rIL28B	0.080	3218.3 - [2445.5, 6368.6]	6146.0 - [5178.3, 7597.8]
E. coli(+) % post rIL28A *	0.053	68.8 - [58.4, 83.7]	95.6 - [85.3, 96.6]
E. coli(+) % post rIL28B *	0.064	62.7 - [60.9, 79.1]	93.8 - [75.7, 96.7]
Rhodamine MFI medium #	**0.047**	349.4 - [305.5, 424.7]	213.3 - [176.7, 312.4]
Rhodamine MFI post rIL29 #	**0.049**	319.2 - [298.7, 431.6]	238.4 - [179.5, 275.4]
Rhodamine MFI post rIL28A #	**0.045**	326.1 - [283.6, 465.8]	241.1 - [167.1, 258.9]
Rhodamine MFI post rIL28B #	0.069	319.2 - [278.1, 448.0]	232.9 - [163.0, 289.1]

* *'E. coli* (+) %' represents neutrophil phagocytic activity.
# 'Rhod MFI' represents neutrophil production of reactive oxygen species.
Unpaired t test was used to analyse group 1 and 2 clinical data (normal data).
Mann-Whitney test was used to analyse group 1 and group 2 experimental data.

IFN-λ1 was not detected by ELISA in the plasma samples or post-*E. coli* supernatant samples.

Incubation of whole blood with IFN-λ1, -λ2 or -λ3 did not alter neutrophil expression of CD11b, CD16, CD182, TLR2 or TLR4. No clear trend was seen on the expression of PSGL-1 or CD62L. Group 1 had higher baseline resting burst than group 2. There were no clear trends seen on phagocytosis or oxidative burst following treatment with IFN-λ1, -λ2 or -λ3. In addition, no changes in pro- and anti-inflammatory cytokine production were observed post-treatment.

6.5 Discussion

After confirming IFN-λr mRNA expression on neutrophils and that neutrophil IFN-λ1 and IFN-λ2/3 mRNA could be induced, the patient study was designed. At this point there was not a single study describing IFN-λr expression or the effect of lambda on neutrophils.

The data reported herein identifies human neutrophils as producers of IFN-λ1 in response to bacterial infection and reveal a previously unknown impairment in neutrophil function in ARC. Marked differences in production of IFN-λ1 in response to *E. coli* were seen within the patient cohort compared to HCs and those who lacked production had significantly more advanced liver disease. Given the magnitude of difference seen this may be of greater significance in a larger cohort. Although the marked impairment in IFN-λ1 production in our patients was seen in the neutrophil fraction, on further analyses the patients who were not seen to induce neutrophil IFN-λ1 in response to *E. coli* also had significantly less induction of leukocyte IFN-λ1. This perhaps supports the concept of cross-talk between neutrophils and the adaptive immune system; more work is required to investigate this finding. It is important to note that neutrophils have low RNA content and so do not constitute the majority of RNA within the leukocyte fraction given the volumes of blood used.

A difference in confirmed infection between the two groups was not seen, this would require a larger study population.

It is difficult to postulate why the profound difference observed in IFN-λ1 between the patient groups was not mirrored in the neutrophil or leucocyte expression of IFN-λ2/3 and IFN-λr expression. On review of the literature, a

complete understanding of the difference between IFN-λ1 and IFN-λ2/3 has not yet been reached. My data would suggest that perhaps the IFN-λ2/3 is of more relevance in the total leukocyte response rather than being neutrophil-specific. Within the patient cohort there was a more variable response in neutrophil IFN-λr expression, and with the study of a larger number of patients this may be of further interest.

There was no correlation between induction of IFN-λ1 and IFN-λr mRNA. In addition, unlike in the HL-60 cells, incubation of whole blood with recombinant IFN-λ did not impact on neutrophil phagocytosis or oxidative burst. This leads me to believe that the IFN-λ1 produced is likely to be biologically active elsewhere, potentially at epithelial surfaces, rather than eliciting an autocrine response. By inference, the lack of production may be an additional factor exacerbating the lack of protection at the gut epithelium and contributing to bacterial translocation which is thought to be a key driver of immunoparesis in advanced liver cirrhosis. This may, in fact, be in conjunction with important effects on the enteric virome. The study of IFN-λ1 at the gut epithelial surface of patients with ALD warrants investigation and may be of particular importance in patients with cirrhosis secondary to the synergistic effects of alcohol and viral hepatitis. An effect on whole blood cytokine production 2 hours post recombinant IFN-λ incubation was not seen. This could be explained by the timeframe used which may be too narrow for the synthesis of *de novo* cytokines or support the concept that IFN-λ is more active at epithelial surfaces. Alternatively, this could support the thought that *in vivo* lambda treatment may

not induce or exacerbate an inflammatory response and this would be of interest to study in larger numbers and using a range of duration of treatment *in vitro*.

Treatment of whole blood with IFN-λ1, -λ2 or -λ3 did not alter neutrophil expression of CD11b, CD16, CD182, TLR2 or TLR4. There was a more variable response in the expression of PSGL-1 and CD62L; these markers influence neutrophil migration and endothelial cell-interaction, and again, this would be useful to study in larger numbers. It would be of interest to examine post-IFN-λ treated lymphocyte and monocyte function, and, in retrospect, the PBMCs could have been collected and stored post-treatment for later analyses which would have added to this study.

The lack of detection of IFN-λ in the second ELISA and particularly the lack in the post *E. coli* supernatants in the HC may be explained by transient presence of the protein, indeed most of the published data refer to mRNA rather than the protein. If pure neutrophil supernatant was analysed this may have given different results. This would require further analyses including patients in a septic phase or recovering from sepsis.

In this study, a cohort of patients with decompensated ARC were examined, two of the patients had superimposed AAH; these two patients, perhaps unsurprisingly, fell into Group 1. Given our findings and the findings of Blazek *et al.* there may be clinical benefit in the analyses of a large cohort of patients with acute severe AAH, the progression to which remains poorly understood with limited treatment options.

In conclusion, these findings confirm an IFN-λ response in non-viral infection, clarify the neutrophil as a producer of IFN-λ and reveal a previously unknown deficient IFN-λ production in ARC which requires further exploration. Elucidation of these mechanisms may have important implications for therapeutic developments in an era of multi-drug resistance and within the spectrum of ALD.

7 General Discussion and Future Work

Here follows a discussion of the most salient results from each section of work. I have included a critique and the limitations of each chapter within each section, and concluded with where I see the focus of future work in ALD.

Neutrophils from AAH patients display increased ROS and lactoferrin release and anti-bacterial activities are dysfunctional (Chapter 3).

Neutrophils were confirmed to be activated in AAH. The novel finding of high circulating levels of lactoferrin seen in AAH compared to ARC supports this and, with larger cohorts, could potentially be used to differentiate those with superimposed AAH from those without the condition. Reduced antibacterial functions were seen across the spectrum of patients I examined. Downstream signaling, for example, post TLR4 activation was not evaluated in these studies and further clarification of the pathways triggered or perhaps inadequately triggered require further investigation. A key question is whether the neutrophils within the liver vasculature behave in a similar way to the peripheral neutrophils; this was not addressed in my work. As discussed in chapter 3 the analyses of liver biopsies may provide some information regarding this, as would analyses within animal models, which are continually being improved [224]. Intravital confocal microscopy, which allows observation of biological processes *in vivo* at high resolution, may also be another mode of exploring this.

The potential for neutrophil elastase to induce bystander tissue damage has been recognised for many years [225]. Given the recent interest in NET formation in liver injury and, in particular, the finding that liver damage was reduced (by

80%) if NET production was blocked [144], this seems a ripe area for further analyses in AAH. A key question that stems from this work is whether AAH indeed has an infective trigger. One way to increase our understanding around this is with longitudinal analyses and the comparison of a large number of patients with pre- or early AAH with a population of heavy alcohol-drinkers who do not develop the condition; only then will some of the protective factors come to light. Neutrophil cell maturity in AAH and apoptotic pathways including 'clear-up' mechanisms are additional areas of research development which may add to the understanding of the pathophysiology in this condition.

AAH T cells express high levels of immune inhibitory receptors, produce lower levels of interferon gamma, and have increased IL-10 production, these effects can be reversed by blocking PD1 and TIM3, which increase the antimicrobial activity of T cells and neutrophils (Chapter 3).

The work on PD1-TIM3, published in Gastroenterology, defined the potential for a new therapeutic, highlighted by the editor [226]. The immunosuppression rather than the initial cytokine storm may be the predominant driver of mortality and research on agents to improve outcome should perhaps focus on this aspect alongside targets to improve regeneration within the liver.

The numbers I studied were unfortunately too small to provide clear-cut outcome correlations, or to allow analysis of the subgroups of prednisolone-treated versus non-prednisolone exposed groups. Neutrophil-TLR4 fold change post-LPS stimulation was, however, significantly lower amongst the 6-month non-survivors compared to the 6-month survivors. This provides further support for endotoxin tolerance and the potential negative impact of this in AAH.

The impact of prednisolone on neutrophil function in AAH (Chapter 4).

A notable finding in my sequential analyses appeared to be the negative impact of steroids on phagocytosis. Vergis *et al.* show that in patients treated with prednisolone, infection exerts an independent effect on mortality by 90 days; furthermore the authors speculate that 7 days of prednisolone therapy may be enough to impair host immunity to allow development of serious infection, and that discontinuation of steroids after 7 days may be unable to reverse the damage [97]. Correlation of bacterial DNA with neutrophil functional assays would be of interest; and my data support the need for further stratification of patients for steroid-prescribing. In summary, the effect of prednisolone on neutrophil function appeared detrimental at an *in vitro* level without the potential benefits of suppressing inflammation, as there was no impact on neutrophil ROS or lactoferrin release.

The differentiated HL-60 cells express many of the key receptors examined in the human study and could be used to further investigate neutrophil dysfunction and the impact of individual drivers in ALD (Chapter 5).

The HL-60 cells could be used in further models, for example, co-culture systems as discussed in chapter 5. The impact of ethanol and hepatocyte-metabolised ethanol on phagocytosis is of interest and perhaps links in with the lower phagocytosis seen in the AA patients compared to the ABA. In a recent review on sepsis in ALD it is stated that the impact of alcoholic aetiology and active alcohol consumption on infection-related short-term mortality in cirrhosis is unclear [227]. Although there have been studies examining the impact of alcohol, both acute and chronic, on bacterial translocation in healthy individuals [228], there

are few studies that have examined direct ethanol effect on the bone marrow. Given the key role that immune cells play in ARC and specifically in AAH this would seem important to further understand. The impact of LPS on the HL-60 cells was not further examined and this is a limitation of this work. No change was seen on HL-60 TLR9 expression following ethanol exposure. Although much more work would be needed to investigate the increased TLR9 I detected in my AAH patients, the lack of induction by ethanol on these cells potentially points towards other factors, such as DAMPs, playing a role, and these cells could be used to examine this.

Endothelial-neutrophil interactions may add to the understanding of why certain individuals develop this condition. In retrospect, it would have been of potential use to have broadened the analyses of the neutrophil cell surface markers to include the selectins, such as PSGL-1, in my initial cohort of patients. This could then have been correlated with the plasma selectin analyses. Interest in gene sequencing of the CXCL family of chemokines in AAH is growing and may ultimately serve as both biomarker and therapeutic target.

Neutrophil-specific production of interferon-λ1 is deficient in advanced alcohol-related liver cirrhosis (Chapter 6).

In my final results chapter the contribution of neutrophil-specific IFN-λ, in anti-bacterial host immune defense is reported. This pathway is strikingly compromised in patients with severe ARC. Little is known about IFN-λ in the context of bacterial infection and the neutrophil-IFN-λ relationship both in health and disease states has been largely unexplored. The findings confirm the

role of IFN-λ in non-viral infection, clarify the neutrophil as a producer of IFN-λ and reveal a previously unknown deficient IFN-λ production in advanced ARC which may have implications for the future advances in the management of this condition. Whilst this study includes a small number of patients, the differences seen are marked and likely to be of greater significance in a larger cohort. Work is ongoing to further recruit AAH patients so that the above results can be built upon, and the manuscript is in preparation. Given the lack of treatment strategies to address bacterial infections, in an era of multi-drug resistance, I believe this work is of wide interest given the new understanding it adds to the field of neutrophil biology and host immunity in bacterial infection. A recent study has demonstrated a dual effect of IFN-λ on both the innate and adaptive arms of the immune response *in vivo* during chronic viral infection [229]. IFN-λ requires further assessment in the different stages of ALD, with increased patient numbers. This will potentially add to the understanding of the link between innate and adaptive immunity in this condition, and the relevance of this on the pathogenenesis of the different clinical stages. Longitudinal study will also deepen understanding of ALD and sepsis susceptibility. Genotyping patients in relation to IFN-λ signaling may also prove to be of clinical relevance in this group. This will allow tailored patient care, more effective use of antimicrobials and further identification of targets for therapy.

A recently published study demonstrate an important role of fungal dysbiosis in the development of ALD [230]; translocation of fungal β-glucan induced liver inflammation via the C-type lectin-like receptor, CLEC7A, on Kupffer cells and, possibly, other bone-marrow derived cells. Subsequent increases in IL-1β

expression and secretion contributed to hepatocyte damage. ARC patients had increased systemic exposure to mycobiota and the levels of exposure and immune response correlated with mortality. It would seem that further work into fungal exposure in the AAH patients is of worth, both in terms of seeking triggers of disease, and, also with regard to improving outcome once patients have developed the condition. If transplantation for this group of patients were to become increasingly practiced, research into this area would be of additional benefit. The study of not just the microbiome but also of the virome and fungal species and interplay between these is of key importance I believe, particularly with regard to the trigger of AAH. IFN-λ may be an important link between these compartments and critical in homeostatic mechanisms.

Summary of results

After confirming the activation of neutrophils and the blunted response to LPS in AAH, the potential for reversibility of this dysfunction was examined. Greater understanding of why certain patients enter a harmful compensatory anti-inflammatory response syndrome is clearly important; and the evolution of disease and impact of prednisolone was studied in Chapter 4. Although more work is needed, the potential negative immunosuppressive effects of prednisolone were highlighted. The individual trajectory within the disease and the influence of endotoxin tolerance was seen. These clinical findings led to further questions, specifically do other derangements in neutrophil function contribute to the state of immunoparesis in ALD and is there a stronger link between this immune compartment and the gut or at epithelial surfaces. The HL-60 cells enabled the exploration of basic neutrophil biology and the exploration

of the lambda receptor led to further questions regarding the relationship between neutrophils and IFN-λ. My final results chapter identifies human neutrophils as producers of IFN-λ1 in response to bacterial infection and reveals a previously unknown impairment in neutrophil function in ARC.

Concluding remarks

In my earlier chapters I have alluded to the difficulty of follow-up of patients which limited interpretation of some results. This would need to be taken into account when designing future studies. To identify triggers of AAH and, also to learn more about moderate AAH, for example those with a Maddrey's score of 20-32 who still have significant mortality at 90 days, large scale studies are required which include excess alcohol drinkers. Studies should not just be limited to those who already have severe AAH. This collaborative approach would also facilitate pharmacologic addiction trials in alcohol use disorder and ALD. I believe that further work on sarcopenia and outcomes including steroid response is of interest, and of particular importance at a time where transplantation for non-responders may be considered. A team approach to wide research aims for this group should be adopted as in clinical practice and, with this, over time, the burden of morbidity and mortality for these patients may be improved. Limitations accepted, I hope that my results have added to the understanding of this condition, which has been, to some extent, neglected for many years. It is pleasing to see that the tide is changing; with increased interest in defining appropriate end-points, collaborative studies and potential novel therapeutic development.

Abstracts and publications related to this work

Publications:

Neutrophil-specific production of interferon-λ1 is deficient in severe alcoholic hepatitis. Manuscript in preparation.

Riva A, Patel V, Kurioka A, Jeffery HC, Wright G, Tarff S, Shawcross DL**, Ryan JM**, Evans A, Azarian S, Bajaj JS, Fagan A, Patel V, Mehta K, Lopez C, Simonova M, Katzarov K, Hadhziolova T, Pavlova S, Wendon JA, Htun Oo Y, Klenerman P, Williams R, Chokshi S. Mucosa-associated invariant T cells link intestinal immunity with antibacterial immune defects in alcoholic liver disease. Gut. 2018 May;67(5):918-930.

Vergis N, Khamri W, Beale K, Sadiq F, Aletrari MO, Moore C, Atkinson SR, Bernsmeier C, Possamai L, Petts G, **Ryan JM**, Foxton M, Hogan B, Foster GR, O'Brien AJ, Ma Y, Shawcross D, Wendon JA, Antoniades CG, Thursz MR. Defective monocyte oxidative burst predicts infection in alcoholic hepatitis and is associated with reduced expression of NADPH oxidase. Gut. 2017 Mar;66(3):519-529.

Manakkat Vijay GK, **Ryan JM,** Abeles RD, Ramage S, Patel V, Bernsmeier C, Riva A, McPhail MJW, Tranah TH, Markwick LJ, Taylor NJ, Bernal W, Auzinger G, Willars C, Chokshi S, Wendon JA, Ma Y, Shawcross DL. Neutrophil Toll-Like Receptor 9 Expression and the Systemic Inflammatory Response in Acetaminophen-Induced Acute Liver Failure. Critical Care Medicine. 2016 Jan;44(1):43-53.

Markwick LJ, Riva A**, Ryan JM**, Cooksley H, Palma E, Tranah T, Manakkat Vijay GK, Vergis N, Thursz M, Evans A, Wright G, Tarff S, O'Grady J, Williams R, Shawcross DL, Chokshi S. Blockade of PD1 and TIM3 restores innate and adaptive immunity in patients with acute alcoholic hepatitis. Gastroenterology. 2015 Mar;148(3):590-602.

Abstracts:

Ryan JM, Riva A, Wright G, Evans A, Shawcross DL, Williams R, Chokshi S. Neutrophil-specific production of interferon-λ1 is deficient in advanced alcohol-related liver cirrhosis. Hepatology. 2016 Volume 64.

Riva A, **Ryan JM**, Tarff S, Wright G, Evans A, Williams R, Shawcross DL, Chokshi S. Frequency and function of anti-bacterial MAIT cells are significantly impaired in advanced alcoholic liver disease. Journal of Hepatology. 2015 Volume 62, s205.

Ryan JM, Manakkat Vijay GK, Abeles RD, Tranah TH, Marwick L, Blackmore LJ, Kronsten VT, Markwick LJ, Riva A, Vergis N, Taylor NJ, Chokshi S, Ma Y, O'Grady J, Shawcross DL. Neutrophil toll-like receptor 9 expression and plasma lactoferrin in acute alcoholic hepatitis and the susceptibility to fungal infection. Hepatology. 2014 Volume 60, p113A.

Blackmore LJ, **Ryan JM,** Manakkat Vijay GK, Huang X, Antoniades CG, Shawcross DL, Ma Y. Patients with acute alcoholic hepatitis present strong Th1 cellular immune response to alcohol dehydrogenase, which are related to impairment of the PD-1/pD-L1 pathway. Hepatology. 2014 Volume 60, p229A.

Manakkat Vijay GK, Vizcay-Barrena G, Tranah TH, Glover L, Patel VC, Bernsmeier C, **Ryan JM**, Blackmore LJ, Huang X, Kronsten VT, Taylor NJ, Bernal W, Auzinger G, Willars C, Wendon J, Ma Y, Bain B, Warley A, Shawcross DL. Increased neutrophil actin production and translocation of myeloperoxidase in patients with acetaminophen-induced acute liver failure may contribute to multiorgan failure. Hepatology. 2014 Volume 60, p558A.

Ryan JM, Manakkat Vijay GK, Abeles RD, Tranah TH, Marwick L, Blackmore LJ, Riva A, Vergis N, Taylor NJ, Chokshi S, Ma Y, O'Grady J, Shawcross DL. The role of neutrophil endotoxin tolerance in the predisposition to sepsis in alcohol-related liver disease. Hepatology 2013 Volume 58, p 312A. **(Oral presentation).**

Ryan JM, Manakkat Vijay GK, Abeles RD, Tranah TH, Marwick L, Riva A, Vergis N, Taylor NJ, O'Grady J, Wendon J, Chokshi S, Shawcross DL. Circulating neutrophil endotoxin tolerance in patients with advanced cirrhosis and acute alcoholic hepatitis impairs appropriate neutrophil responses to an infectious challenge. EASL Monothematic Conference, Bacterial Infections in Cirrhosis, May 2013.

Markwick LJ, **Ryan JM**, Cooksley H, Williams R, Evans A, Shawcross DL, Chokshi S. Restoration of anti-bacterial innate and adaptive immunity in patients with acute alcoholic hepatitis via blockade of T-cell specific immunoinhibitory pathways. EASL Monothematic Conference, Bacterial Infections in Cirrhosis, May 2013.

Manakkat Vijay GK, Barrena GV, Glover L, **Ryan JM**, Tranah T, Ma Y, Bain B, Warley A, Shawcross DL. Hyperlactoferrinemia in acute alcoholic hepatitis and susceptibility to infection. EASL Monothematic Conference, Bacterial Infections in Cirrhosis, May 2013.

Vergis N, Khamri W, Tidswell R, Mullish B, Kabir M, **Ryan JM**, Antoniades C, Thursz M. Phenotypic and functional modulation of circulating monocyte subsets may increase predisposition to infection in cirrhosis and alcoholic hepatitis. EASL Monothematic Conference, Bacterial Infections in Cirrhosis, May 2013.

Ryan JM, Manakkat Vijay GK, Tranah TH, Marwick L, Abeles RD, Vergis N, Blackmore LJ, Taylor NJ, Chokshi S, O'Grady J, Shawcross DL. Down-regulation of toll-like receptor -2 and -4 in circulating neutrophils in response to systemic oxidative stress may contribute to increased susceptibility to infection in alcoholic hepatitis. Journal of Hepatology. 2013 Volume 58, p S223. **(Oral presentation).**

Markwick LJ, **Ryan JM**, Palma E, Cooksley H, Tranah TH, Vergis N, Williams R, Evans A, Shawcross DL, Chokshi S. Susceptibility to infection in patients with acute alcoholic hepatitis: a novel role for PD-1 and Galectin-9? Journal of Hepatology. 2013 Volume 58, p S221.

Tranah TH, Manakkat Vijay GK, **Ryan JM**, Abeles RD, Markwick L, Wendon JA, Chana PJ, Shawcross DL. Circulating neutrophil granule subset release in response to bacterial stimulus is augmented in patients with alcohol-related liver disease and may contribute to organ bystander damage. Journal of Hepatology. 2013 Volume 58, p S225.

Ryan JM, Taylor NJ, Manakkat Vijay GK, Tranah TH, Abeles RD, Riva A, Vergis N, Markwick L, Chokshi S, O'Grady J, Wendon JA, Ma Y, Shawcross DL. Alcohol – Always detrimental to the immune system? The role of active alcohol consumption on neutrophil function in alcohol-related cirrhosis. Gut. 2012 Volume 61, (Supplement No. 2), p A200.

Tranah T, Manakkat Vijay GK, **Ryan JM**, Abeles RD, Taylor NJ, Vergis N, Markwick L, Wendon JA, O'Grady J, Riva A, Chokshi S, Ma Y, Shawcross DL. Active alcohol consumption induces functional immune paresis but paradoxically promotes endotoxin tolerance in those with advanced alcohol-related cirrhosis. Gut. 2012 Volume 61, (Supplement No. 2), p A416.

Manakkat Vijay GK, Abeles RD, Ramage S, Riva A, Verghis N, **Ryan JM**, McPhail M, Taylor NJ, Wendon J, Chokshi S, Ma Y, Shawcross DL. Neutrophil intracellular Toll-like receptor (TLR) 9 expression serves as a biomarker that determines the presence and severity of encephalopathy in acute liver failure and cirrhosis. Journal of Hepatology. 2012 Volume 56, (Supplement No. 2), p S102.

Manakkat Vijay GK, Abeles RD, Ramage SN, Riva A, **Ryan JM**, McPhail M, Nishtala A, Taylor NJ, Wendon J, Chokshi S, Ma Y, Shawcross DL. Upregulation of neutrophil intracellular Toll-like receptor (TLR) 9 expression by elevated arterial ammonia in patients with acute and chronic liver failure determines response to endotoxin and bacterial challenge. Hepatology. 2011 Volume 54 (Supplement No. 4), p 477A.

Manakkat Vijay GK, Abeles RD, Ramage SN, Riva A, **Ryan JM**, McPhail M, Nishtala A, Taylor NJ, Wendon J, Chokshi S, Ma Y, Shawcross DL. Role of circulating neutrophil Toll-like receptor (TLR) 4 expression and endotoxin sensitivity in acute acetaminophen-induced liver failure in predicting the response to bacterial and ammonia. Hepatology. 2011 Volume 54 (Supplement No. 4), p 498A.

References

1. Williams, R., et al., *Addressing liver disease in the UK: a blueprint for attaining excellence in health care and reducing premature mortality from lifestyle issues of excess consumption of alcohol, obesity, and viral hepatitis.* Lancet, 2014. **384**(9958): p. 1953-97.
2. Desborough, M.J., et al., *Patterns of blood component use in cirrhosis: a nationwide study.* Liver Int, 2015.
3. Sheron, N., et al., *Projections of alcohol-related deaths in England and Wales--tragic toll or potential prize?* Lancet, 2012. **379**(9817): p. 687-8.
4. Sheron, N., *Alcohol and liver disease in Europe--Simple measures have the potential to prevent tens of thousands of premature deaths.* J Hepatol, 2016. **64**(4): p. 957-67.
5. O'Shea, R.S., S. Dasarathy, and A.J. McCullough, *Alcoholic liver disease.* Hepatology, 2010. **51**(1): p. 307-28.
6. Teli, M.R., et al., *Determinants of progression to cirrhosis or fibrosis in pure alcoholic fatty liver.* Lancet, 1995. **346**(8981): p. 987-90.
7. Orman, E.S., G. Odena, and R. Bataller, *Alcoholic liver disease: pathogenesis, management, and novel targets for therapy.* J Gastroenterol Hepatol, 2013. **28 Suppl 1**: p. 77-84.
8. Mancebo, A., et al., *Annual incidence of hepatocellular carcinoma among patients with alcoholic cirrhosis and identification of risk groups.* Clin Gastroenterol Hepatol, 2013. **11**(1): p. 95-101.
9. Crabb, D.W., et al., *Standard Definitions and Common Data Elements for Clinical Trials in Patients With Alcoholic Hepatitis: Recommendation From the NIAAA Alcoholic Hepatitis Consortia.* Gastroenterology, 2016. **150**(4): p. 785-90.
10. *EASL clinical practical guidelines: management of alcoholic liver disease.* J Hepatol, 2012. **57**(2): p. 399-420.
11. Verma, S., et al., *Prevalence of septic events, type 1 hepatorenal syndrome, and mortality in severe alcoholic hepatitis and utility of discriminant function and MELD score in predicting these adverse events.* Dig Dis Sci, 2006. **51**(9): p. 1637-43.
12. Gustot, T., et al., *Invasive aspergillosis in patients with severe alcoholic hepatitis.* J Hepatol, 2014. **60**(2): p. 267-74.
13. Louvet, A., et al., *Infection in patients with severe alcoholic hepatitis treated with steroids: early response to therapy is the key factor.* Gastroenterology, 2009. **137**(2): p. 541-8.
14. Lucey, M.R., P. Mathurin, and T.R. Morgan, *Alcoholic hepatitis.* N Engl J Med, 2009. **360**(26): p. 2758-69.
15. Thursz, M. and T.R. Morgan, *Treatment of Severe Alcoholic Hepatitis.* Gastroenterology, 2016. **150**(8): p. 1823-34.
16. Forrest, E.H. and D. Gleeson, *Is a liver biopsy necessary in alcoholic hepatitis?* J Hepatol, 2012. **56**(6): p. 1427-8; author reply 1428-9.
17. Kryger, P., et al., *The accuracy of the clinical diagnosis in acute hepatitis and alcoholic liver disease. Clinical versus morphological diagnosis.* Scand J Gastroenterol, 1983. **18**(5): p. 691-6.

18. Mookerjee, R.P., et al., *The role of liver biopsy in the diagnosis and prognosis of patients with acute deterioration of alcoholic cirrhosis.* J Hepatol, 2011. **55**(5): p. 1103-11.
19. Dhanda, A.D., P.L. Collins, and C.A. McCune, *Is liver biopsy necessary in the management of alcoholic hepatitis?* World J Gastroenterol, 2013. **19**(44): p. 7825-9.
20. Hardy, T., et al., *White cell count and platelet count associate with histological alcoholic hepatitis in jaundiced harmful drinkers.* BMC Gastroenterol, 2013. **13**(1): p. 55.
21. Altamirano, J., et al., *A histologic scoring system for prognosis of patients with alcoholic hepatitis.* Gastroenterology, 2014. **146**(5): p. 1231-9 e1-6.
22. Pugh, R.N., et al., *Transection of the oesophagus for bleeding oesophageal varices.* Br J Surg, 1973. **60**(8): p. 646-9.
23. Christensen, E., et al., *Prognostic value of Child-Turcotte criteria in medically treated cirrhosis.* Hepatology, 1984. **4**(3): p. 430-5.
24. Poynard, T., et al., *Prognostic value of total serum bilirubin/gamma-glutamyl transpeptidase ratio in cirrhotic patients.* Hepatology, 1984. **4**(2): p. 324-7.
25. Infante-Rivard, C., S. Esnaola, and J.P. Villeneuve, *Clinical and statistical validity of conventional prognostic factors in predicting short-term survival among cirrhotics.* Hepatology, 1987. **7**(4): p. 660-4.
26. Gines, P., et al., *Compensated cirrhosis: natural history and prognostic factors.* Hepatology, 1987. **7**(1): p. 122-8.
27. Ochs, A., et al., *The transjugular intrahepatic portosystemic stent-shunt procedure for refractory ascites.* N Engl J Med, 1995. **332**(18): p. 1192-7.
28. Cooper, G.S., et al., *A prognostic model for patients with end-stage liver disease.* Gastroenterology, 1997. **113**(4): p. 1278-88.
29. Rajan, D.K., Z.J. Haskal, and T.W. Clark, *Serum bilirubin and early mortality after transjugular intrahepatic portosystemic shunts: results of a multivariate analysis.* J Vasc Interv Radiol, 2002. **13**(2 Pt 1): p. 155-61.
30. Malinchoc, M., et al., *A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts.* Hepatology, 2000. **31**(4): p. 864-71.
31. Kamath, P.S., et al., *A model to predict survival in patients with end-stage liver disease.* Hepatology, 2001. **33**(2): p. 464-70.
32. Wiesner, R., et al., *Model for end-stage liver disease (MELD) and allocation of donor livers.* Gastroenterology, 2003. **124**(1): p. 91-6.
33. Sheth, M., M. Riggs, and T. Patel, *Utility of the Mayo End-Stage Liver Disease (MELD) score in assessing prognosis of patients with alcoholic hepatitis.* BMC Gastroenterol, 2002. **2**: p. 2.
34. Dunn, W., et al., *MELD accurately predicts mortality in patients with alcoholic hepatitis.* Hepatology, 2005. **41**(2): p. 353-8.
35. Srikureja, W., et al., *MELD score is a better prognostic model than Child-Turcotte-Pugh score or Discriminant Function score in patients with alcoholic hepatitis.* J Hepatol, 2005. **42**(5): p. 700-6.
36. Maddrey, W.C., et al., *Corticosteroid therapy of alcoholic hepatitis.* Gastroenterology, 1978. **75**(2): p. 193-9.

37. Carithers, R.L., Jr., et al., *Methylprednisolone therapy in patients with severe alcoholic hepatitis. A randomized multicenter trial.* Ann Intern Med, 1989. **110**(9): p. 685-90.
38. Mendenhall, C.L., et al., *Short-term and long-term survival in patients with alcoholic hepatitis treated with oxandrolone and prednisolone.* N Engl J Med, 1984. **311**(23): p. 1464-70.
39. Forrest, E.H., et al., *Analysis of factors predictive of mortality in alcoholic hepatitis and derivation and validation of the Glasgow alcoholic hepatitis score.* Gut, 2005. **54**(8): p. 1174-9.
40. Forrest, E.H., et al., *The Glasgow alcoholic hepatitis score identifies patients who may benefit from corticosteroids.* Gut, 2007. **56**(12): p. 1743-6.
41. Louvet, A., et al., *The Lille model: a new tool for therapeutic strategy in patients with severe alcoholic hepatitis treated with steroids.* Hepatology, 2007. **45**(6): p. 1348-54.
42. Dominguez, M., et al., *A new scoring system for prognostic stratification of patients with alcoholic hepatitis.* Am J Gastroenterol, 2008. **103**(11): p. 2747-56.
43. Papastergiou, V., et al., *Nine scoring models for short-term mortality in alcoholic hepatitis: cross-validation in a biopsy-proven cohort.* Aliment Pharmacol Ther, 2014. **39**(7): p. 721-32.
44. Palaniyappan, N., et al., *The utility of scoring systems in predicting early and late mortality in alcoholic hepatitis: whose score is it anyway?* Int J Hepatol, 2012. **2012**: 624675.
45. Louvet, A., et al., *Combining Data From Liver Disease Scoring Systems Better Predicts Outcomes of Patients With Alcoholic Hepatitis.* Gastroenterology, 2015. **149**(2): p. 398-406 e8; quiz e16-7.
46. Potts, J.R., et al., *Determinants of long-term outcome in severe alcoholic hepatitis.* Aliment Pharmacol Ther, 2013. **38**(6): p. 584-95.
47. Louvet, A., et al., *Main drivers of outcome differ between short term and long term in severe alcoholic hepatitis: A prospective study.* Hepatology, 2017. **66**(5): p. 1464-1473.
48. Louvet, A. and P. Mathurin, *Alcoholic liver disease: mechanisms of injury and targeted treatment.* Nat Rev Gastroenterol Hepatol, 2015. **12**(4): p. 231-42.
49. Luedde, T., N. Kaplowitz, and R.F. Schwabe, *Cell death and cell death responses in liver disease: mechanisms and clinical relevance.* Gastroenterology, 2014. **147**(4): p. 765-783 e4.
50. Petrasek, J., et al., *STING-IRF3 pathway links endoplasmic reticulum stress with hepatocyte apoptosis in early alcoholic liver disease.* Proc Natl Acad Sci U S A, 2013. **110**(41): p. 16544-9.
51. Ji, C. and N. Kaplowitz, *Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice.* Gastroenterology, 2003. **124**(5): p. 1488-99.
52. Mathurin, P., et al., *Exacerbation of alcoholic liver injury by enteral endotoxin in rats.* Hepatology, 2000. **32**(5): p. 1008-17.
53. Parlesak, A., et al., *Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease.* J Hepatol, 2000. **32**(5): p. 742-7.

54. Fujimoto, M., et al., *Plasma endotoxin and serum cytokine levels in patients with alcoholic hepatitis: relation to severity of liver disturbance.* Alcohol Clin Exp Res, 2000. **24**(4 Suppl): p. 48S-54S.
55. Hanck, C., et al., *Presence of plasma endotoxin is correlated with tumour necrosis factor receptor levels and disease activity in alcoholic cirrhosis.* Alcohol Alcohol, 1998. **33**(6): p. 606-8.
56. Lu, Y.C., W.C. Yeh, and P.S. Ohashi, *LPS/TLR4 signal transduction pathway.* Cytokine, 2008. **42**(2): p. 145-51.
57. Gao, B. and R. Bataller, *Alcoholic liver disease: pathogenesis and new therapeutic targets.* Gastroenterology, 2011. **141**(5): p. 1572-85.
58. Afford, S.C., et al., *Distinct patterns of chemokine expression are associated with leukocyte recruitment in alcoholic hepatitis and alcoholic cirrhosis.* J Pathol, 1998. **186**(1): p. 82-9.
59. Colmenero, J., et al., *Hepatic expression of candidate genes in patients with alcoholic hepatitis: correlation with disease severity.* Gastroenterology, 2007. **132**(2): p. 687-97.
60. Maltby, J., et al., *Chemokine levels in human liver homogenates: associations between GRO alpha and histopathological evidence of alcoholic hepatitis.* Hepatology, 1996. **24**(5): p. 1156-60.
61. Taieb, J., et al., *Blood neutrophil functions and cytokine release in severe alcoholic hepatitis: effect of corticosteroids.* J Hepatol, 2000. **32**(4): p. 579-86.
62. Mandal, P., et al., *The anti-inflammatory effects of adiponectin are mediated via a heme oxygenase-1-dependent pathway in rat Kupffer cells.* Hepatology, 2010. **51**(4): p. 1420-9.
63. Wan, J., et al., *M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease.* Hepatology, 2014. **59**(1): p. 130-42.
64. Byun, J.S., et al., *Activation of toll-like receptor 3 attenuates alcoholic liver injury by stimulating Kupffer cells and stellate cells to produce interleukin-10 in mice.* J Hepatol, 2013. **58**(2): p. 342-9.
65. Schnabl, B. and D.A. Brenner, *Interactions between the intestinal microbiome and liver diseases.* Gastroenterology, 2014. **146**(6): p. 1513-24.
66. Malaguarnera, G., et al., *Gut microbiota in alcoholic liver disease: pathogenetic role and therapeutic perspectives.* World J Gastroenterol, 2014. **20**(44): p. 16639-48.
67. Llopis, M., et al., *Intestinal microbiota contributes to individual susceptibility to alcoholic liver disease.* Gut, 2016. **65**(5): p. 830-9.
68. Gao, B., et al., *Innate immunity in alcoholic liver disease.* Am J Physiol Gastrointest Liver Physiol, 2011. **300**(4): p. G516-25.
69. Seki, E., et al., *TLR4 enhances TGF-beta signaling and hepatic fibrosis.* Nat Med, 2007. **13**(11): p. 1324-32.
70. Palsson-McDermott, E.M. and L.A. O'Neill, *Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4.* Immunology, 2004. **113**(2): p. 153-62.
71. Jaeschke, H., *Neutrophil-mediated tissue injury in alcoholic hepatitis.* Alcohol, 2002. **27**(1): p. 23-7.

72. Albano, E., *Role of adaptive immunity in alcoholic liver disease.* Int J Hepatol, 2012. **2012**: 893026.
73. Keir, M.E., et al., *PD-1 and its ligands in tolerance and immunity.* Annu Rev Immunol, 2008. **26**: p. 677-704.
74. Evans, A., et al., *Programmed death 1 expression during antiviral treatment of chronic hepatitis B: Impact of hepatitis B e-antigen seroconversion.* Hepatology, 2008. **48**(3): p. 759-69.
75. Han, G., et al., *Tim-3: an activation marker and activation limiter of innate immune cells.* Front Immunol, 2013. **4**: p. 449.
76. McMahan, R.H., et al., *Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity.* J Clin Invest, 2010. **120**(12): p. 4546-57.
77. Fourcade, J., et al., *Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients.* J Exp Med, 2010. **207**(10): p. 2175-86.
78. Markwick, L.J., et al., *Blockade of PD1 and TIM3 restores innate and adaptive immunity in patients with acute alcoholic hepatitis.* Gastroenterology, 2015. **148**(3): p. 590-602 e10.
79. Stickel, F., et al., *The genetics of alcohol dependence and alcohol-related liver disease.* J Hepatol, 2017. **66**(1): p. 195-211.
80. Whitfield, J.B., et al., *Brief report: genetics of alcoholic cirrhosis-GenomALC multinational study.* Alcohol Clin Exp Res, 2015. **39**(5): p. 836-42.
81. Jung, Y., et al., *Accumulation of hedgehog-responsive progenitors parallels alcoholic liver disease severity in mice and humans.* Gastroenterology, 2008. **134**(5): p. 1532-43.
82. Sancho-Bru, P., et al., *Liver progenitor cell markers correlate with liver damage and predict short-term mortality in patients with alcoholic hepatitis.* Hepatology, 2012. **55**(6): p. 1931-41.
83. Odena, G., et al., *LPS-TLR4 Pathway Mediates Ductular Cell Expansion in Alcoholic Hepatitis.* Sci Rep, 2016. **6**: p. 35610.
84. Dubuquoy, L., et al., *Progenitor cell expansion and impaired hepatocyte regeneration in explanted livers from alcoholic hepatitis.* Gut, 2015. **64**(12): p. 1949-60.
85. Lieber, C.S., D.P. Jones, and L.M. Decarli, *EFFECTS OF PROLONGED ETHANOL INTAKE: PRODUCTION OF FATTY LIVER DESPITE ADEQUATE DIETS.* J Clin Invest, 1965. **44**: p. 1009-21.
86. Lieber, C.S., L.M. DeCarli, and M.F. Sorrell, *Experimental methods of ethanol administration.* Hepatology, 1989. **10**(4): p. 501-10.
87. Bertola, A., O. Park, and B. Gao, *Chronic plus binge ethanol feeding synergistically induces neutrophil infiltration and liver injury in mice: a critical role for E-selectin.* Hepatology, 2013. **58**(5): p. 1814-23.
88. Mathews, S., et al., *Animals models of gastrointestinal and liver diseases. Animal models of alcohol-induced liver disease: pathophysiology, translational relevance, and challenges.* Am J Physiol Gastrointest Liver Physiol, 2014. **306**(10): p. G819-23.
89. Fernandez, J., et al., *Bacterial infections in cirrhosis: epidemiological changes with invasive procedures and norfloxacin prophylaxis.* Hepatology, 2002. **35**(1): p. 140-8.

90. Borzio, M., et al., *Bacterial infection in patients with advanced cirrhosis: a multicentre prospective study.* Dig Liver Dis, 2001. **33**(1): p. 41-8.
91. Fernandez, J., et al., *Prevalence and risk factors of infections by multiresistant bacteria in cirrhosis: a prospective study.* Hepatology, 2012. **55**(5): p. 1551-61.
92. Gustot, T., et al., *Severe sepsis in cirrhosis.* Hepatology, 2009. **50**(6): p. 2022-33.
93. Rajkovic, I.A. and R. Williams, *Abnormalities of neutrophil phagocytosis, intracellular killing and metabolic activity in alcoholic cirrhosis and hepatitis.* Hepatology, 1986. **6**(2): p. 252-62.
94. Jalan, R., et al., *Bacterial infections in cirrhosis: a position statement based on the EASL Special Conference 2013.* J Hepatol, 2014. **60**(6): p. 1310-24.
95. Foreman, M.G., D.M. Mannino, and M. Moss, *Cirrhosis as a risk factor for sepsis and death: analysis of the National Hospital Discharge Survey.* Chest, 2003. **124**(3): p. 1016-20.
96. Arvaniti, V., et al., *Infections in patients with cirrhosis increase mortality four-fold and should be used in determining prognosis.* Gastroenterology, 2010. **139**(4): p. 1246-56, 1256 e1-5.
97. Vergis, N., et al., *In Patients With Severe Alcoholic Hepatitis, Prednisolone Increases Susceptibility to Infection and Infection-Related Mortality, and Is Associated With High Circulating Levels of Bacterial DNA.* Gastroenterology, 2017. **152**(5): p. 1068-1077 e4.
98. Thursz, M.R., et al., *Prednisolone or pentoxifylline for alcoholic hepatitis.* N Engl J Med, 2015. **372**(17): p. 1619-28.
99. Gustot, T., et al., *Impact of infection on the prognosis of critically ill cirrhotic patients: results from a large worldwide study.* Liver Int, 2014. **34**(10): p. 1496-503.
100. Mathurin, P., et al., *Early liver transplantation for severe alcoholic hepatitis.* N Engl J Med, 2011. **365**(19): p. 1790-800.
101. Theocharidou, E., et al., *Early invasive fungal infections and colonization in patients with cirrhosis admitted to the intensive care unit.* Clin Microbiol Infect, 2016. **22**(2): p. 189 e1-7.
102. West, K.A., et al., *Granulocyte transfusions in the management of invasive fungal infections.* Br J Haematol, 2017. **177**(3): p. 357-374.
103. Chaulk, J., et al., *Third-generation cephalosporin-resistant spontaneous bacterial peritonitis: a single-centre experience and summary of existing studies.* Can J Gastroenterol Hepatol, 2014. **28**(2): p. 83-8.
104. Tandon, P., et al., *High prevalence of antibiotic-resistant bacterial infections among patients with cirrhosis at a US liver center.* Clin Gastroenterol Hepatol, 2012. **10**(11): p. 1291-8.
105. Fernandez, J., et al., *Antibiotic prophylaxis in cirrhosis: Good and bad.* Hepatology, 2016. **63**(6): p. 2019-31.
106. Bone, R.C., et al., *Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine.* Chest, 1992. **101**(6): p. 1644-55.
107. Bone, R.C., C.J. Grodzin, and R.A. Balk, *Sepsis: a new hypothesis for pathogenesis of the disease process.* Chest, 1997. **112**(1): p. 235-43.

108. Tschaikowsky, K., et al., *Coincidence of pro- and anti-inflammatory responses in the early phase of severe sepsis: Longitudinal study of mononuclear histocompatibility leukocyte antigen-DR expression, procalcitonin, C-reactive protein, and changes in T-cell subsets in septic and postoperative patients.* Crit Care Med, 2002. **30**(5): p. 1015-23.
109. Monneret, G., et al., *The anti-inflammatory response dominates after septic shock: association of low monocyte HLA-DR expression and high interleukin-10 concentration.* Immunol Lett, 2004. **95**(2): p. 193-8.
110. Cazzaniga, M., et al., *The systemic inflammatory response syndrome in cirrhotic patients: relationship with their in-hospital outcome.* J Hepatol, 2009. **51**(3): p. 475-82.
111. Albillos, A., M. Lario, and M. Alvarez-Mon, *Cirrhosis-associated immune dysfunction: distinctive features and clinical relevance.* J Hepatol, 2014. **61**(6): p. 1385-96.
112. Michelena, J., et al., *Systemic inflammatory response and serum lipopolysaccharide levels predict multiple organ failure and death in alcoholic hepatitis.* Hepatology, 2015. **62**(3): p. 762-72.
113. Rorvig, S., et al., *Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors.* J Leukoc Biol, 2013. **94**(4): p. 711-21.
114. Kruger, P., et al., *Neutrophils: Between host defence, immune modulation, and tissue injury.* PLoS Pathog, 2015. **11**(3): p. e1004651.
115. Bratton, D.L. and P.M. Henson, *Neutrophil clearance: when the party is over, clean-up begins.* Trends Immunol, 2011. **32**(8): p. 350-7.
116. Stark, M.A., et al., *Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17.* Immunity, 2005. **22**(3): p. 285-94.
117. Pillay, J., et al., *In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days.* Blood, 2010. **116**(4): p. 625-7.
118. Summers, C., et al., *Neutrophil kinetics in health and disease.* Trends Immunol, 2010. **31**(8): p. 318-24.
119. Potts, J.R., et al., *Circulating granulocyte lifespan in compensated alcohol-related cirrhosis: a pilot study.* Physiol Rep, 2016. **4**(17).
120. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
121. Ley, K., et al., *Getting to the site of inflammation: the leukocyte adhesion cascade updated.* Nat Rev Immunol, 2007. **7**(9): p. 678-89.
122. Dale, D.C., L. Boxer, and W.C. Liles, *The phagocytes: neutrophils and monocytes.* Blood, 2008. **112**(4): p. 935-45.
123. Weiss, S.J., *Tissue destruction by neutrophils.* N Engl J Med, 1989. **320**(6): p. 365-76.
124. Selsted, M.E., et al., *Primary structures of three human neutrophil defensins.* J Clin Invest, 1985. **76**(4): p. 1436-9.
125. Borregaard, N., *Neutrophils, from marrow to microbes.* Immunity, 2010. **33**(5): p. 657-70.
126. Brock, J.H., *Lactoferrin--50 years on.* Biochem Cell Biol, 2012. **90**(3): p. 245-51.
127. McCulloh, R. and S.M. Opal, *Human recombinant lactoferrin for sepsis: too good to be true?* Crit Care Med, 2013. **41**(3): p. 908-9.

128. Guntupalli, K., et al., *A phase 2 randomized, double-blind, placebo-controlled study of the safety and efficacy of talactoferrin in patients with severe sepsis.* Crit Care Med, 2013. **41**(3): p. 706-16.
129. Futosi, K., S. Fodor, and A. Mocsai, *Neutrophil cell surface receptors and their intracellular signal transduction pathways.* Int Immunopharmacol, 2013. **17**(3): p. 638-50.
130. Bruhns, P., *Properties of mouse and human IgG receptors and their contribution to disease models.* Blood, 2012. **119**(24): p. 5640-9.
131. Coxon, A., et al., *Fc gamma RIII mediates neutrophil recruitment to immune complexes. a mechanism for neutrophil accumulation in immune-mediated inflammation.* Immunity, 2001. **14**(6): p. 693-704.
132. Schymeinsky, J., A. Mocsai, and B. Walzog, *Neutrophil activation via beta2 integrins (CD11/CD18): molecular mechanisms and clinical implications.* Thromb Haemost, 2007. **98**(2): p. 262-73.
133. Takeishi, Y. and I. Kubota, *Role of Toll-like receptor mediated signaling pathway in ischemic heart.* Front Biosci (Landmark Ed), 2009. **14**: p. 2553-8.
134. Vaure, C. and Y. Liu, *A comparative review of toll-like receptor 4 expression and functionality in different animal species.* Front Immunol, 2014. **5**: p. 316.
135. Park, B.S., et al., *The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex.* Nature, 2009. **458**(7242): p. 1191-5.
136. Lizundia, R., et al., *Host species-specific usage of the TLR4-LPS receptor complex.* Innate Immun, 2008. **14**(4): p. 223-31.
137. Brinkmann, V., et al., *Neutrophil extracellular traps kill bacteria.* Science, 2004. **303**(5663): p. 1532-5.
138. Urban, C.F., et al., *Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans.* PLoS Pathog, 2009. **5**(10): p. e1000639.
139. Nauseef, W.M. and N. Borregaard, *Neutrophils at work.* Nat Immunol, 2014. **15**(7): p. 602-11.
140. Xu, R., et al., *The role of neutrophils in the development of liver diseases.* Cell Mol Immunol, 2014. **11**(3): p. 224-31.
141. Jaeschke, H. and T. Hasegawa, *Role of neutrophils in acute inflammatory liver injury.* Liver Int, 2006. **26**(8): p. 912-9.
142. Adams, D.H., et al., *Mechanisms of immune-mediated liver injury.* Toxicol Sci, 2010. **115**(2): p. 307-21.
143. Degre, D., et al., *Hepatic expression of CCL2 in alcoholic liver disease is associated with disease severity and neutrophil infiltrates.* Clin Exp Immunol, 2012. **169**(3): p. 302-10.
144. Kolaczkowska, E., et al., *Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature.* Nat Commun, 2015. **6**: p. 6673.
145. Rajkovic, I.A. and R. Williams, *Mechanisms of abnormalities in host defences against bacterial infection in liver disease.* Clin Sci (Lond), 1985. **68**(3): p. 247-53.
146. Fiuza, C., et al., *In vivo neutrophil dysfunction in cirrhotic patients with advanced liver disease.* J Infect Dis, 2000. **182**(2): p. 526-33.

147. Tritto, G., et al., *Evidence of neutrophil functional defect despite inflammation in stable cirrhosis.* J Hepatol, 2011. **55**(3): p. 574-81.
148. Ono, Y., et al., *Opsonophagocytic dysfunction in patients with liver cirrhosis and low responses to tumor necrosis factor-alpha and lipopolysaccharide in patients' blood.* J Infect Chemother, 2004. **10**(4): p. 200-7.
149. Rolas, L., et al., *Inhibition of mammalian target of rapamycin aggravates the respiratory burst defect of neutrophils from decompensated patients with cirrhosis.* Hepatology, 2013. **57**(3): p. 1163-71.
150. Garfia, C., I. Garcia-Ruiz, and J.A. Solis-Herruzo, *Deficient phospholipase C activity in blood polimorphonuclear neutrophils from patients with liver cirrhosis.* J Hepatol, 2004. **40**(5): p. 749-56.
151. Fiuza, C., et al., *Granulocyte colony-stimulating factor improves deficient in vitro neutrophil transendothelial migration in patients with advanced liver disease.* Clin Diagn Lab Immunol, 2002. **9**(2): p. 433-9.
152. Taylor, N.J., et al., *The severity of circulating neutrophil dysfunction in patients with cirrhosis is associated with 90-day and 1-year mortality.* Aliment Pharmacol Ther, 2014. **40**(6): p. 705-15.
153. Shawcross, D.L., et al., *Ammonia impairs neutrophil phagocytic function in liver disease.* Hepatology, 2008. **48**(4): p. 1202-12.
154. Mookerjee, R.P., et al., *Neutrophil dysfunction in alcoholic hepatitis superimposed on cirrhosis is reversible and predicts the outcome.* Hepatology, 2007. **46**(3): p. 831-40.
155. Stadlbauer, V., et al., *Role of Toll-like receptors 2, 4, and 9 in mediating neutrophil dysfunction in alcoholic hepatitis.* Am J Physiol Gastrointest Liver Physiol, 2009. **296**(1): p. G15-22.
156. Gallin, J.I., *Human neutrophil heterogeneity exists, but is it meaningful?* Blood, 1984. **63**(5): p. 977-83.
157. Collins, S.J., R.C. Gallo, and R.E. Gallagher, *Continuous growth and differentiation of human myeloid leukaemic cells in suspension culture.* Nature, 1977. **270**(5635): p. 347-9.
158. Sachs, L., *The differentiation of myeloid leukaemia cells: new possibilities for therapy.* Br J Haematol, 1978. **40**(4): p. 509-17.
159. Collins, S.J., *The HL-60 promyelocytic leukemia cell line: proliferation, differentiation, and cellular oncogene expression.* Blood, 1987. **70**(5): p. 1233-44.
160. Fleck, R.A., S. Romero-Steiner, and M.H. Nahm, *Use of HL-60 cell line to measure opsonic capacity of pneumococcal antibodies.* Clin Diagn Lab Immunol, 2005. **12**(1): p. 19-27.
161. Le Cabec, V., J. Calafat, and N. Borregaard, *Sorting of the specific granule protein, NGAL, during granulocytic maturation of HL-60 cells.* Blood, 1997. **89**(6): p. 2113-21.
162. Fleck, R.A., et al., *Optimization of nb-4 and hl-60 differentiation for use in opsonophagocytosis assays.* In Vitro Cell Dev Biol Anim, 2003. **39**(5-6): p. 235-42.
163. Pantelic, M., et al., *Retinoic acid treated HL60 cells express CEACAM1 (CD66a) and phagocytose Neisseria gonorrhoeae.* FEMS Immunol Med Microbiol, 2004. **42**(2): p. 261-6.

164. Nordenfelt, P., et al., *Phagocytosis of Streptococcus pyogenes by all-trans retinoic acid-differentiated HL-60 cells: roles of azurophilic granules and NADPH oxidase.* PLoS One, 2009. **4**(10): p. e7363.
165. Isaacs, A. and J. Lindenmann, *Virus interference. I. The interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 258-67.
166. Chelbi-Alix, M.K. and J. Wietzerbin, *Interferon, a growing cytokine family: 50 years of interferon research.* Biochimie, 2007. **89**(6-7): p. 713-8.
167. Zhang, S.Y., et al., *Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense.* Immunol Rev, 2008. **226**: p. 29-40.
168. Wheelock, E.F. and W.A. Sibley, *CIRCULATING VIRUS, INTERFERON AND ANTIBODY AFTER VACCINATION WITH THE 17-D STRAIN OF YELLOW-FEVER VIRUS.* N Engl J Med, 1965. **273**: p. 194-8.
169. Sheppard, P., et al., *IL-28, IL-29 and their class II cytokine receptor IL-28R.* Nat Immunol, 2003. **4**(1): p. 63-8.
170. Kotenko, S.V., et al., *IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex.* Nat Immunol, 2003. **4**(1): p. 69-77.
171. Uze, G. and D. Monneron, *IL-28 and IL-29: newcomers to the interferon family.* Biochimie, 2007. **89**(6-7): p. 729-34.
172. Lazear, H.M., T.J. Nice, and M.S. Diamond, *Interferon-lambda: Immune Functions at Barrier Surfaces and Beyond.* Immunity, 2015. **43**(1): p. 15-28.
173. Neuman, M.G., et al., *Alcoholic liver disease: Clinical and translational research.* Exp Mol Pathol, 2015. **99**(3): p. 596-610.
174. Tilg, H., et al., *Serum levels of cytokines in chronic liver diseases.* Gastroenterology, 1992. **103**(1): p. 264-74.
175. Tiegs, G., *Cellular and cytokine-mediated mechanisms of inflammation and its modulation in immune-mediated liver injury.* Z Gastroenterol, 2007. **45**(1): p. 63-70.
176. Lemmers, A., et al., *The interleukin-17 pathway is involved in human alcoholic liver disease.* Hepatology, 2009. **49**(2): p. 646-57.
177. Egli, A., et al., *The impact of the interferon-lambda family on the innate and adaptive immune response to viral infections.* Emerg Microbes Infect, 2014. **3**(7): p. e51.
178. Hermant, P. and T. Michiels, *Interferon-lambda in the context of viral infections: production, response and therapeutic implications.* J Innate Immun, 2014. **6**(5): p. 563-74.
179. Galmozzi, E., M. Vigano, and P. Lampertico, *Systematic review with meta-analysis: do interferon lambda 3 polymorphisms predict the outcome of interferon-therapy in hepatitis B infection?* Aliment Pharmacol Ther, 2014. **39**(6): p. 569-78.
180. Wolk, K., et al., *Maturing dendritic cells are an important source of IL-29 and IL-20 that may cooperatively increase the innate immunity of keratinocytes.* J Leukoc Biol, 2008. **83**(5): p. 1181-93.
181. Osterlund, P., et al., *Gene expression and antiviral activity of alpha/beta interferons and interleukin-29 in virus-infected human myeloid dendritic cells.* J Virol, 2005. **79**(15): p. 9608-17.

182. Coccia, E.M., et al., *Viral infection and Toll-like receptor agonists induce a differential expression of type I and lambda interferons in human plasmacytoid and monocyte-derived dendritic cells.* Eur J Immunol, 2004. **34**(3): p. 796-805.
183. Blazek, K., et al., *IFN-lambda resolves inflammation via suppression of neutrophil infiltration and IL-1beta production.* J Exp Med, 2015. **212**(6): p. 845-53.
184. Bell, H., et al., *Long-term prognosis of patients with alcoholic liver cirrhosis: a 15-year follow-up study of 100 Norwegian patients admitted to one unit.* Scand J Gastroenterol, 2004. **39**(9): p. 858-63.
185. Shawcross, D.L. and J.G. O'Grady, *The 6-month abstinence rule in liver transplantation.* Lancet, 2010. **376**(9737): p. 216-7.
186. Rice, J.P. and M.R. Lucey, *Should length of sobriety be a major determinant in liver transplant selection?* Curr Opin Organ Transplant, 2013. **18**(3): p. 259-64.
187. Lucey, M.R., et al., *Effect of alcoholic liver disease and hepatitis C infection on waiting list and posttransplant mortality and transplant survival benefit.* Hepatology, 2009. **50**(2): p. 400-6.
188. Morgan, M.Y., *The treatment of alcoholic hepatitis.* Alcohol Alcohol, 1996. **31**(2): p. 117-34.
189. Rambaldi, A., et al., *Systematic review: glucocorticosteroids for alcoholic hepatitis--a Cochrane Hepato-Biliary Group systematic review with meta-analyses and trial sequential analyses of randomized clinical trials.* Aliment Pharmacol Ther, 2008. **27**(12): p. 1167-78.
190. Mathurin, P., et al., *Corticosteroids improve short-term survival in patients with severe alcoholic hepatitis: meta-analysis of individual patient data.* Gut, 2011. **60**(2): p. 255-60.
191. Singh, S., et al., *Comparative Effectiveness of Pharmacological Interventions for Severe Alcoholic Hepatitis: A Systematic Review and Network Meta-analysis.* Gastroenterology, 2015. **149**(4): p. 958-70 e12.
192. Mathurin, P., et al., *Survival and prognostic factors in patients with severe alcoholic hepatitis treated with prednisolone.* Gastroenterology, 1996. **110**(6): p. 1847-53.
193. Ramond, M.J., et al., *A randomized trial of prednisolone in patients with severe alcoholic hepatitis.* N Engl J Med, 1992. **326**(8): p. 507-12.
194. Mendenhall, C.L., et al., *VA cooperative study on alcoholic hepatitis. II: Prognostic significance of protein-calorie malnutrition.* Am J Clin Nutr, 1986. **43**(2): p. 213-8.
195. Fialla, A.D., et al., *Nutritional therapy in cirrhosis or alcoholic hepatitis: a systematic review and meta-analysis.* Liver Int, 2015. **35**(9): p. 2072-8.
196. Moreno, C., et al., *Intensive Enteral Nutrition Is Ineffective for Patients With Severe Alcoholic Hepatitis Treated With Corticosteroids.* Gastroenterology, 2016. **150**(4): p. 903-10 e8.
197. Akriviadis, E., et al., *Pentoxifylline improves short-term survival in severe acute alcoholic hepatitis: a double-blind, placebo-controlled trial.* Gastroenterology, 2000. **119**(6): p. 1637-48.
198. Whitfield, K., et al., *Pentoxifylline for alcoholic hepatitis.* Cochrane Database Syst Rev, 2009(4): p. CD007339.

199. Lebrec, D., et al., *Pentoxifylline does not decrease short-term mortality but does reduce complications in patients with advanced cirrhosis.* Gastroenterology, 2010. **138**(5): p. 1755-62.
200. Mathurin, P., et al., *Prednisolone with vs without pentoxifylline and survival of patients with severe alcoholic hepatitis: a randomized clinical trial.* JAMA, 2013. **310**(10): p. 1033-41.
201. Tilg, H., et al., *Anti-tumor necrosis factor-alpha monoclonal antibody therapy in severe alcoholic hepatitis.* J Hepatol, 2003. **38**(4): p. 419-25.
202. Naveau, S., et al., *A double-blind randomized controlled trial of infliximab associated with prednisolone in acute alcoholic hepatitis.* Hepatology, 2004. **39**(5): p. 1390-7.
203. Boetticher, N.C., et al., *A randomized, double-blinded, placebo-controlled multicenter trial of etanercept in the treatment of alcoholic hepatitis.* Gastroenterology, 2008. **135**(6): p. 1953-60.
204. Nguyen-Khac, E., et al., *Glucocorticoids plus N-acetylcysteine in severe alcoholic hepatitis.* N Engl J Med, 2011. **365**(19): p. 1781-9.
205. Singh, V., et al., *Granulocyte colony-stimulating factor in severe alcoholic hepatitis: a randomized pilot study.* Am J Gastroenterol, 2014. **109**(9): p. 1417-23.
206. Brown, M. and C. Wittwer, *Flow cytometry: principles and clinical applications in hematology.* Clin Chem, 2000. **46**(8 Pt 2): p. 1221-9.
207. Subrahmanyam, Y.V., et al., *RNA expression patterns change dramatically in human neutrophils exposed to bacteria.* Blood, 2001. **97**(8): p. 2457-68.
208. Mandrekar, P., et al., *Alcoholic hepatitis: Translational approaches to develop targeted therapies.* Hepatology, 2016. **64**(4): p. 1343-55.
209. Okubo, K., et al., *Lactoferrin Suppresses Neutrophil Extracellular Traps Release in Inflammation.* EBioMedicine, 2016. **10**: p. 204-15.
210. Tranah, T.H., et al., *Dysfunctional neutrophil effector organelle mobilization and microbicidal protein release in alcohol-related cirrhosis.* Am J Physiol Gastrointest Liver Physiol, 2017. **313**(3): p. G203-G211.
211. Manakkat Vijay, G.K., et al., *Neutrophil Toll-Like Receptor 9 Expression and the Systemic Inflammatory Response in Acetaminophen-Induced Acute Liver Failure.* Crit Care Med, 2016. **44**(1): p. 43-53.
212. Clark, R.A., J.I. Gallin, and A.S. Fauci, *Effects of in vivo prednisone on in vitro eosinophil and neutrophil adherence and chemotaxis.* Blood, 1979. **53**(4): p. 633-41.
213. Mann, B.S. and K.F. Chung, *Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy.* Respir Res, 2006. **7**: p. 59.
214. Saffar, A.S., H. Ashdown, and A.S. Gounni, *The molecular mechanisms of glucocorticoids-mediated neutrophil survival.* Curr Drug Targets, 2011. **12**(4): p. 556-62.
215. Haziot, A., B.Z. Tsuberi, and S.M. Goyert, *Neutrophil CD14: biochemical properties and role in the secretion of tumor necrosis factor-alpha in response to lipopolysaccharide.* J Immunol, 1993. **150**(12): p. 5556-65.
216. Jasek, E., J. Mirecka, and J.A. Litwin, *Effect of differentiating agents (all-trans retinoic acid and phorbol 12-myristate 13-acetate) on drug sensitivity of HL60 and NB4 cells in vitro.* Folia Histochem Cytobiol, 2008. **46**(3): p. 323-30.

217. Mita, Y., et al., *Induction of Toll-like receptor 4 in granulocytic and monocytic cells differentiated from HL-60 cells.* Br J Haematol, 2001. **112**(4): p. 1041-7.
218. Gacouin, A., et al., *Acute alcohol exposure has an independent impact on C-reactive protein levels, neutrophil CD64 expression, and subsets of circulating white blood cells differentiated by flow cytometry in nontrauma patients.* Shock, 2014. **42**(3): p. 192-8.
219. Zhang, P., et al., *Acute ethanol intoxication suppresses the pulmonary inflammatory response in rats challenged with intrapulmonary endotoxin.* Alcohol Clin Exp Res, 1997. **21**(5): p. 773-8.
220. Sabino, K.R., A. Petroianu, and L.R. Alberti, *Influence of the acute alcoholism on the phagocytic function of the mononuclear phagocytic system.* J Med Life, 2011. **4**(4): p. 421-3.
221. Chiu, C.H., et al., *Influence of ethanol concentration in the phagocytic function of neutrophils against Klebsiella pneumoniae isolates in an experimental model.* J Microbiol Immunol Infect, 2016.
222. Shuto, T., et al., *Increased responsiveness to TLR2 and TLR4 ligands during dimethylsulfoxide-induced neutrophil-like differentiation of HL-60 myeloid leukemia cells.* Leuk Res, 2007. **31**(12): p. 1721-8.
223. Spellberg, B.J., et al., *A phagocytic cell line markedly improves survival of infected neutropenic mice.* J Leukoc Biol, 2005. **78**(2): p. 338-44.
224. Gao, B., et al., *Animal Models of Alcoholic Liver Disease: Pathogenesis and Clinical Relevance.* Gene Expr, 2017. **17**(3): p. 173-186.
225. Janoff, A., *Elastase in tissue injury.* Annu Rev Med, 1985. **36**: p. 207-16.
226. Bataller, R. and P. Mandrekar, *Identifying molecular targets to improve immune function in alcoholic hepatitis.* Gastroenterology, 2015. **148**(3): p. 498-501.
227. Gustot, T., et al., *Sepsis in alcohol-related liver disease.* J Hepatol, 2017.
228. Bala, S., et al., *Acute binge drinking increases serum endotoxin and bacterial DNA levels in healthy individuals.* PLoS One, 2014. **9**(5): p. e96864.
229. Phillips, S., et al., *Peg-Interferon Lambda Treatment Induces Robust Innate and Adaptive Immunity in Chronic Hepatitis B Patients.* Front Immunol, 2017. **8**: p. 621.
230. Yang, A.M., et al., *Intestinal fungi contribute to development of alcoholic liver disease.* J Clin Invest, 2017. **127**(7): p. 2829-2841.

Appendices

Antibodies used for characterisation of neutrophil phenotype

Antibody (Fluorochrome)	Clone	Reactivity	Supplier	Concentration	Dilution
CD16 (PE)	3G8	Human	BD Biosciences, UK	100 Tests	1:500
CD11b (APC-Cy7)	ICRF44	Human	BD Biosciences, UK	100 Tests	1:500
CD282 (Alexafluor 488)	11G7	Human	BD Biosciences, UK	100 Tests	1:500
TLR4 (Biotin-conjugated)	HTA125	Human	BD Biosciences, UK	100 Tests	1:500
Streptavidin (PE-Cy7)	--	Human	BD Biosciences, UK	0.2 mg/mL	1:500
TLR9 (APC)	eB72-1665	Human	BD Biosciences, UK	100 Tests	1:500

Ethanol-treated HL-60 phagocytosis

Cells were spun down at 250 g for 5 minutes and seeded to 1 x10^6 cells/mL in their old culture media and 100 μL added per tube (total cell number per tube 1 x 10^5). Ethanol (10, 50, 100, 250, 500 mM) or 100 μL of supernatant (control 24 hr, EtOH 24 hr) was added to the cells and incubated for 90 minutes at 37°C/5% CO_2. After this time, 50 μL *E.coli*-FITC was added and cells incubated at 37°C/5% CO_2 for 240 minutes, prior to being washed twice and then analyzed on the FACS canto II.

Acknowledgements

I would like to thank my supervisor Dr Debbie Shawcross for her unwavering support and help, without which I would not have been able to complete this thesis. I would like to thank Dr Shilpa Chokshi at the Foundation for Liver Research, she allowed me to develop my skills as a researcher and gave me opportunity to pursue my area of research, her support has been invaluable. I would like to thank my second supervisor Professor John O'Grady.

I thank Godhev Mannakat Vijay who helped me immensely.

I thank Antonio Riva for his guidance.

I thank my family, particularly my beautiful, kind Mum and my ever-supportive sister, Julie. I thank my friends who have listened to me, particularly Alice.

Lastly and most importantly I would like to thank the patients for agreeing to be in my study and hope that this work in some way will improve treatments for similar individuals in the future.